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FK506 and the Role of Immunophilins in Nerve Regeneration

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Abstract

FK506 is a new FDA-approved immunosuppressant used for prevention of allograft rejection in, for example, liver and kidney transplantations. FK506 is inactive by itself and requires binding to an FK506 binding protein-12 (FKBP-12), or immunophilin, for activation. In this regard, FK506 is analogous to cyclosporin A, which must bind to its immunophilin (cyclophilin A) to display activity. This FK506-FKBP complex inhibits the activity of the serine/threonine protein phosphatase 2B (calcineurin), the basis for the immunosuppressant action of FK506. The discovery that immunophilins are also present in the nervous system introduces a new level of complexity in the regulation of neuronal function. Two important calcineurin targets in brain are the growth-associated protein GAP-43 and nitric oxide (NO) synthase (NOS).

This review focuses on studies showing that systemic administration of FK506 dose-dependently speeds nerve regeneration and functional recovery in rats following a sciatic-nerve crush injury. The effect appears to result from an increased rate of axonal regeneration. The nerve regenerative property of this class of agents is separate from their immunosuppressant action because FK506-related compounds that bind to FKBP-12 but do not inhibit calcineurin are also able to increase nerve regeneration. Thus, FK506's ability to increase nerve regeneration arises via a calcineurin-independent mechanism (i.e., one not involving an increase in GAP-43 phosphorylation). Possible mechanisms of action are discussed in relation to known actions of FKBPs: the interaction of FKBP-12 with two Ca^{2+} release-channels (the ryanodine and inositol 1,4,5-triphosphate receptors) which is disrupted by FK506, thereby increasing Ca^{2+} flux; the type 1 receptor for the transforming growth factor-β (TGF-β1), which stimulates nerve growth factor (NGF) synthesis by glial cells, and is a natural ligand for FKBP-12; and the immunophilin FKBP-52/FKBP-59, which has also been identified as a heat-shock protein (HSP-56) and is a component of the nontransformed glucocorticoid receptor.

Taken together, studies of FK506 indicate broad functional roles for the immunophilins in the nervous system. Both calcineurin-dependent (e.g., neuroprotection via reduced NO formation) and calcineurin-independent mechanisms (i.e., nerve regeneration) need to be invoked to explain the many different neuronal effects of FK506. This suggests that multiple immunophilins mediate FK506's neuronal effects. Novel, nonimmunosuppressant ligands for FKBPs may represent important new drugs for the treatment of a variety of neurological disorders.

Index Entries: Calcineurin; cyclosporin A; FK506; FKBP-12; glucocorticoid receptor; immunophilin; immunosuppressant; nerve regeneration.

The Current Status of Drugs for Nerve Regeneration

The development of new therapeutics to augment nerve regeneration is an area of intense research activity. Some of the agents that have been experimentally examined are the melanocortins, adrenocorticotrophic hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH) (Bijlsma et al., 1981; 1984; De Koning and Gispen, 1987a; Edwards et al., 1985; Sporel-Özakat et al., 1990; Strand and Kung, 1980; Strand and Smith, 1986), the trisubstituted ACTH analog Org 2766 (Sporel-Özakat et al., 1990; De Koning and Gispen, 1987b), testosterone (Jones, 1993), uridine in experimental diabetic neuropathy (Gallai et al., 1992), gangliosides in diabetic animals (Ekström and Tomlinson, 1990), insulin and insulinlike growth factor (Ekström et al., 1989; Kange et al., 1989; Roth et al., 1995), isaxonine (Sebille et al., 1982), and the long chain fatty alcohol *n*-hexacosanol (Starr et al., 1996). Limited clinical trials have been reported with several of these agents. The most widely lauded of these are the gangliosides. However, in those countries where clinical trials have been undertaken (e.g., Britain, Italy, but not the United States), the use of gangliosides has been discouraged or banned because of the development of Guillain-Barré syndrome in a small percentage of patients (Schonhofer, 1991; Figueras et al., 1992; Landi et al., 1993). Similarly, isaxonine (Nerfactor), was withdrawn from clinical trials because of hepatotoxicity (Letteron et al., 1984). Org 2766 has been tried in a limited number of patients, the results to-date being equivocal (Gorio et al., 1993). One agent currently in clinical use that has been shown to speed functional recovery following a sciatic-nerve crush lesion is nimodipine (Gispen et al., 1991), a dihydropyridine calcium antagonist. However, results obtained from animal studies on its regenerative properties have not been impressive; although regeneration begins slightly earlier, ultimately, there was no difference from untreated animals in the time needed to achieve full recovery (Angelov et al., 1996). Furthermore, the use of this agent for the treatment of nerve regeneration could be limited by possible untoward cardiovascular effects (Murad, 1990). Thus, the search continues for an agent that can be used to enhance nerve regeneration in humans without adverse side effects.

FK506: Historical Background

FK506 is a new FDA-approved immunosuppressant macrolide drug isolated from *Streptomyces tsukubaensis* (Kino et al., 1987a,b) and used for organ transplantations (Hoffman et al., 1990; Starzl et al., 1987, 1989). The drug was discovered and isolated in 1984 from a soil sample obtained from Tsukuba, Japan, upon screening microbial fermentation broths using a mixed lymphocyte reaction (Kino and Goto, 1993). The first experimental study of FK506 on cardiac transplantation in rats was reported in 1987 by Ochiai and coworkers (Ochiai et al., 1987). Clinical trials, pioneered by Thomas Starzl's group at the University of Pittsburgh began in February 1989 (Starzl et al., 1987). Over the years, FK506 has been shown to possess two important properties that make it superior to cyclosporin A, currently the most widely employed drug for preventing allograft rejection. First, FK506 is a more potent immunosuppressant (by approx 10X) than cyclosporin A in vitro (Kino et al., 1987a,b; Tocci et al., 1989), in animals (Ochiai et al., 1987; Murase et al., 1987; Ochiai et al., 1988; Sakai et al., 1991; Todo et al., 1987, 1988, 1989), and in humans, leading to fewer instances of rejection and retransplantation (Hoffman et al., 1990; Starzl et al., 1989; McDiarmid et al., 1995). Second, initial reports indicated that its toxicity in humans was far less than that associated with cyclosporin A (Starzl et al., 1989; Shapiro et al., 1990). Whereas the incidence of toxicity has subsequently been found to vary among transplant centers (Klintmalm, 1994; Neuhaus et al., 1994), including the development of moderate-to-severe neurotoxicity (including cortical blindness, tremor, seizures,

and encephalopathy) in anywhere from 3–21% of patients (Lopez et al., 1991; Mueller et al., 1994; Wijdicks et al., 1994; Bronster et al., 1995; Vincenti et al., 1996), this discrepancy has been attributed to the tendency to overdose with FK506 (Fung et al., 1996). Consequently, FK506 is beginning to replace cyclosporin A as the drug of choice in the treatment of allograft rejection.

FK506: Overview of Neuronal Properties

Whereas its immunosuppressive effects alone make this an important new clinical drug, FK506 has also been found to possess a variety of neuronal properties, including protection against ischemic brain injury (Sharkey and Butcher, 1994; Ide et al., 1996; Tokime et al., 1996; Butcher et al., 1997) and glutamate neurotoxicity in vitro (Dawson et al., 1993) (although a very recent study [Butcher et al., 1997] did not substantiate protection against glutamate toxicity in vivo), prevention of *N*-methyl-D-aspartate (NMDA)-receptor desensitization (Tong et al., 1995), prevention of kindling (Moriwake et al., 1996) blockade of long-term potentiation (LTP) and long-term depression (LTD) in the visual cortex (Torii et al., 1995; Funauchi et al., 1994), facilitation of LTP (Ikegami et al., 1996) and blockade of LTD in the rat hippocampus (Hodgkiss and Kelly, 1995), and alteration in neurotransmitter release (Steiner et al., 1996) and endocytosis (Kuromi et al., 1997). In regard to the latter, whereas LTP and LTD have not been proven to underlie learning and memory; it may be relevant that cyclosporin A also inhibits memory formation in day-old chicks (Bennett et al., 1996). It is likely that all these FK506 neuronal properties are mediated by calcineurin inhibition. For example, calcineurin is known to regulate NMDA-receptor desensitization (*see* Tong et al., 1995). Furthermore, by preventing calcineurin-dependent NOS dephosphorylation, FK506 would inhibit NOS activity thereby reducing formation of NO that has been implicated in mediating neurotoxicity (Dawson et al., 1993; Snyder and Sabatini, 1995), and the generation of both LTP (Schuman and Madison, 1991; O'Dell et al., 1991; Kantor et al., 1996) and LTD (Shibuki and Okada, 1996; Malen and Chapman, 1997). The apparent contradictory findings that FK506 elicits both an inhibition of NMDA-induced neurotransmitter release (Steiner et al., 1996) and an augmentation of depolarization-induced neurotransmitter release have been attributed to a similar underlying mechanism; i.e., altered phosphorylation of the calcineurin substrates NOS and synapsin I, respectively (Steiner et al., 1996). It is possible that any one of these alterations may play a role in the development of some of the neurological sequelae observed in the occasional patient undergoing FK506 therapy (Lopez et al., 1991; Mueller et al., 1994; Wijdicks et al., 1994; Bronster et al., 1995; Vincenti et al., 1996).

In addition to these interesting neuronal properties, FK506 speeds nerve regeneration in the peripheral nervous system of the rat with a focal crush lesion of the sciatic nerve (Gold et al., 1994, 1995). The nerve regenerative property of FK506 is the focus of this review. *A priori,* it would not be expected that an immunosuppressant drug (FK506) would alter axonal regeneration since Wallerian degeneration is not an immune-mediated event (Griffin et al., 1993). Furthermore, even if a reduction in macrophage infiltration (Brück and Friede, 1990) occurs following FK506 administration, such an alteration would be expected to impair nerve regeneration by delaying the removal of products of Wallerian degeneration from the distal stump (Beuche and Friede, 1984; Friede and Brück, 1993) studies utilizing the Ola (Wld) mouse mutant (Perry et al., 1990a,b), for example, show that a delay in Wallerian degeneration and macrophage infiltration leads to an impairment in axonal regeneration of sensory (Ludwin and Bisby, 1992) and motor (Chen and Bisby, 1992) neurons. Why, therefore, should FK506 alter nerve regeneration? To answer this question, it is first

necessary to understand FK506's mechanism of immunosuppression.

The Immunophilins and Immunosuppression

Immunosuppressant drugs FK506 and cyclos-porin A are prodrugs that are activated when bound to their respective binding proteins (immunophilins), FK506-binding-protein (FKBP) and cyclophilin, respectively (Schreiber and Crabtree, 1992). The function of the immunophilins as mediators of immunosuppressant drugs has recently been reviewed (Schreiber, 1991). FKBPs are a family of immunophilin proteins named according to their size (in kD): for example, FKBP-12, -13, -25, -51, -52, and -59 (Schreiber and Crabtree, 1992; Alnemri et al., 1993; Sigal and Dumont, 1992). Initially, it was thought that the immunosuppressant actions of FK506 and cyclosporin A were caused by the ability of their respective immunophilins to prevent the interconversion of the *cis-* and *trans-* isomers of prolyl residues of proteins (Walshet al., 1992). However, subsequent studies proved that peptidyl prolyl isomerase (PPI) activity does not play a role in immunosuppression (Dumont et al., 1992; Wiederrecht et al., 1992), since not all immunosuppressant analogs of these two agents inhibit isomerase activity (Sigal et al., 1991) and, conversely, not all isomerase inhibitors are immunosuppressants (Tocci et al., 1989; Dumont et al., 1992; Sigal et al., 1991; Bierer et al., 1990; for review, *see* Wiederrect and Etzkorn, 1995). [The rotamase activity of the immunophilins has been shown to have physiological functions, such as inhibition of collagen assembly by cyclosporin A (Lewin, 1995; Compton et al., 1992; Bächinger et al., 1993) and FK506 (Hans Peter Bachinger, personal communication).] It is widely accepted that the most likely mechanism for the action of these agents involves inhibition of the activity of *calcineurin* (Freman et al., 1992), a calcium/calmodulin-dependent phosphoserine/phosphothreonine protein phosphatase, also known as PP-2B (Walsh et al., 1992; Dumont et al., 1992; Liu et al., 1991). By inhibiting the calcineurin-induced dephosphorylation of a nuclear factor of activated T-cells (NFAT), FK506 and cyclosporin A prevent NFAT translocation into the nucleus where it induces interleukin-2 (IL-2) secretion, thereby preventing T-cell proliferation (Tocci et al., 1989; Schreiber and Crabtree, 1992; Sigal and Dumont, 1992; Freman et al., 1992; Terada et al., 1992). Of the variety of FKBPs, it has been demonstrated (Sigal and Dumont, 1992; Liu et al., 1991) that FKBP-12 mediates FK506's immunosuppressant activity in T-cells. Whether the inhibition of the expression of the proto-oncogenes *c-myc* and *c-fos* (Sigal and Dumont, 1992) is involved in their immunosuppressant action is unclear. Recently, a second FKBP (FKBP-51), which is specifically expressed in T-lymphocytes (T-cells), has been demonstrated to also inhibit calcineurin (Baughman et al., 1995) suggesting that multiple immunophilins may participate in mediating FK506's immunosuppressant action.

The Immunophilins in the Nervous System

Immunophilins are enriched in neurons throughout the central (Steiner et al., 1992) and peripheral (Lyons et al., 1995) nervous systems. Their distributions and putative actions in regulating neuronal function have also been reviewed (*see* Snyder and Sabatini, 1995; Wiederrect and Etzkorn, 1995; Sánchez and Ning, 1996). Most studied is FKBP-12 (Harding et al., 1989; Siekierka et al., 1989), a ubiquitous protein that has been highly conserved throughout evolution (Siekierka et al., 1990). Snyder and coworkers, who first reported that FKBP-12 is present in neuronal tissue, showed that the mRNA levels for FKBP-12 increase in motor (facial) neurons following peripheral nerve axotomy (Lyons et al., 1992). In addition, calcineurin is present not only in T-cells but also in brain (Steiner

et al., 1992) where it comprises up to 1% of total protein in some brain regions (Klee, 1991). Furthermore, the high levels (relative to other tissues) of this protein in brain (Mukai et al., 1993) correspond regionally with the presence of high levels of FKBP (Steiner et al., 1992). Interestingly, one of the major targets for calcineurin in neurons is the growth-associated protein GAP-43 (Skene, 1990). Both GAP-43 (Skene, 1989; Skene and Willard, 1981a,b) and neuronal phosphatases (Bixby and Jhabvala, 1993) are concentrated in growth cones where they are believed to play an important role in nerve regeneration. Most importantly, a preliminary report (Steiner et al., 1991) indicates that FK506 increases phosphorylation of GAP-43. Based upon this information, FK506 (and, if this model is correct, cyclosporin A) should alter nerve regeneration, perhaps via phosphorylation-dependent activation of GAP-43 (Liu and Storm, 1990). Although this hypothesis initially appeared tenable, the most recent findings from my laboratory (summarized in the next three sections) do not support a calcineurin-dependent mechanism for how FK506 increases the rate of axonal regeneration.

FK506 Increases the Rate of Axonal Regeneration in the Rat Sciatic Nerve

We reported preliminarily in 1993 (Gold et al., 1993) and formally in 1994 (Gold et al., 1994), that FK506 increases functional recovery and nerve regeneration in young adult Sprague-Dawley rats (160–225 g) given a focal sciatic nerve crush (axotomy). A second paper, in 1995, showed that FK506 increases the rate of axonal regeneration in this model (Gold et al., 1995). Axotomy was performed by crushing the nerve twice (for a total of 30 s using a No. 7 Dumont jeweler's forceps) at the level of the hip; the crush site was marked by a sterile 9-0 suture inserted in the epineurial sheath (Gold et al., 1994; 1995). Daily subcutaneous injections of FK506 (1 mg/kg) reduced (by approx 1.5 d) the number of days until the onset of an ability to right the foot and move the toes ("onset"), and the number of days until the animal demonstrated an ability to walk on its hind feet and toes ("walking") (Fig. 1); animals were observed by at least two independent investigators in a double-blinded study (i.e., the treatment regimen was unknown to both the presenter and the observers of the animals). Control (saline- or vehicle-treated) animals first demonstrated an ability to right the foot between 17 and 18 d. In contrast, three of the five FK506-treated animals walked almost normally by 18 days. These differences in functional recovery were reflected in the morphological appearance of the sciatic nerve and its branches distal to the nerve crush at 18 d after axotomy. Axonal calibers (determined by electron microscopy in plastic-embedded sections from glutaraldehyde-perfused animals) for the largest 30% of regenerating axons were increased by 93% in the tibial nerve branch to the soleus muscle from FK506-treated animals (*see* Fig. 3 in Gold et al., 1994). Axons were found to have advanced further toward their targets and reinnervation of intrafusal fibers was evident in the most distal (interosseus) muscles. The maximal distance of axonal elongation from the crush site was measured at 10 and 15 d following axotomy using radiolabeling techniques (Gold et al., 1995), the rate of regeneration being estimated from the slope of the resultant line determined between these two-time points. We found that a 1 mg/kg daily dosage of FK506 significantly ($p < 0.05$) increased (by 16%) the axonal regeneration rate from 3.8 mm/d in saline-treated rats to 4.4 mm/d in FK506-treated animals (*see* Fig. 7 in Gold et al., 1994); regeneration rate was determined using radiolabeling techniques that provide a more accurate and less biased assessment of regenerative distance than the more commonly used pinch test. Taken together, these initial studies demonstrated that FK506 speeds functional recovery by increasing the rate of axonal regeneration in the rat sciatic nerve.

We have recently extended these studies by examining the dose-dependency for FK506's

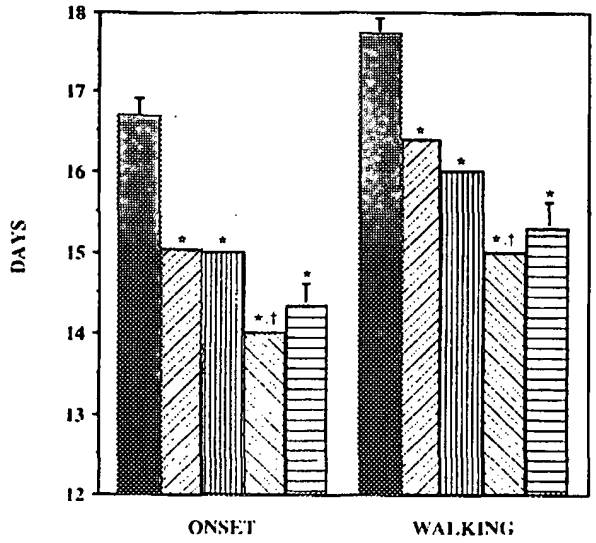

Fig. 1. Bar graphs showing number of days from axotomy until onset of toe movement and an ability to right the foot (left), and an ability to walk on the hind feet (right) are present in saline-treated and FK506-treated rats. Both signs of functional recovery appear earlier in all FK506-treated groups compared to saline-treated controls, being present earliest in the animals given the 5 mg/kg dose. *$p < 0.0001$, compared to saline-treated controls (by one-way ANOVA and Fisher's post-hoc test); †$p < 0.01$, compared to 1 and 2 mg/kg groups (by one-way ANOVA and Fisher's post-hoc test); ††$p < 0.001$, compared to 1 and 2 mg/kg groups (by one-way ANOVA and Fisher's post-hoc test).

nerve regenerative effect (Wang et al., 1997). For these studies, we employed daily subcutaneous injections of FK506 at dosages of 2, 5, or 10 mg/kg. In all our analyses, the best results were obtained in animals given the 5 mg/kg dose. In terms of functional recovery, the 5 mg/kg FK506-treated group demonstrated the earliest recovery of function in the hindfeet (Fig. 1); for example, the number of days until the onset of an ability to right the foot and move the toes ("onset"), and the number of days until the animal demonstrated an ability to walk on its hind feet and toes ("walking") were reduced from 16.8 ± 0.20 d to 14 ± 0 d and 17.8 ± 0.2 d to 15 ± 0 d, respectively, in the saline-treated animals (n = 5) and in the

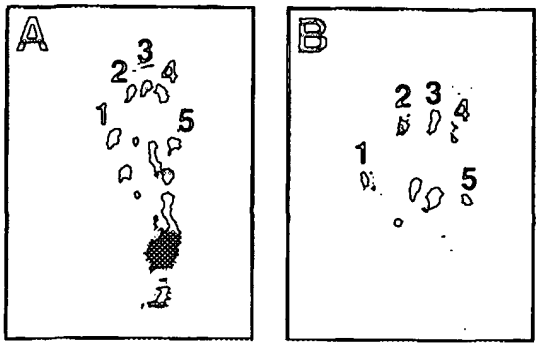

Fig. 2. Representative footprints at 18 d following axotomy from a saline-treated rat **(A)** and an animal given 5 mg/kg FK506 **(B)**. Each image was generated by scanning the original footprint using MacImage (Xerox Imaging Systems, Stamford, CT). The foot and all toes (numbered) are clumped together in the footprint from the saline-treated rat. In contrast, the footprint from the FK506-treated animal exhibits toe spread for all five digits; the lack of an imprint by the heel shows that the animal was able to support its weight on its toes and the front of its foot during walking.

5 mg/kg group (n = 3), respectively. Representative footprints at 18 d following axotomy (corresponding to the time of morphological analysis; see below) are shown in Fig. 2. Whereas the footprint from the saline-treated, axotomized control animal shows a continued deficit (Fig. 2A), the footprint from the animal given the 5 mg/kg dose (Fig. 2B) appears virtually normal; this rat demonstrates an ability to walk on toes and the front of its foot, as shown by the lack of a heel imprint. Electron microscopy, performed at 18 d, confirmed the behavioral findings. Nerves from FK506-treated animals contained larger, more advanced regenerating axons, representing the morphological correlate of the earlier functional recovery in these animals. The percent increase in mean axonal areas from control values demonstrated a bell-shaped dose-dependency (Fig. 3); mean axonal areas in FK506-treated animals (including our earlier data from rats treated with 1 mg/kg/d) were increased by 67, 83, 120, and 100% in the 1, 2, 5, and 10 mg/kg groups, respectively. Measurement of axonal regenera-

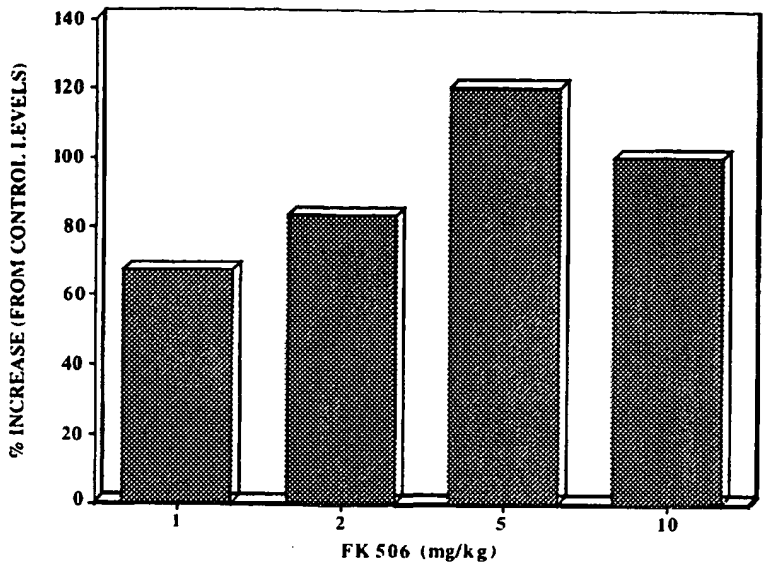

Fig. 3. FK506 dose-dependently increases mean axonal area in the soleus nerve. The percent increase in axonal areas from control values increases with dose between 1, 2, and 5 mg/kg, declining slightly in the 10 mg/kg group.

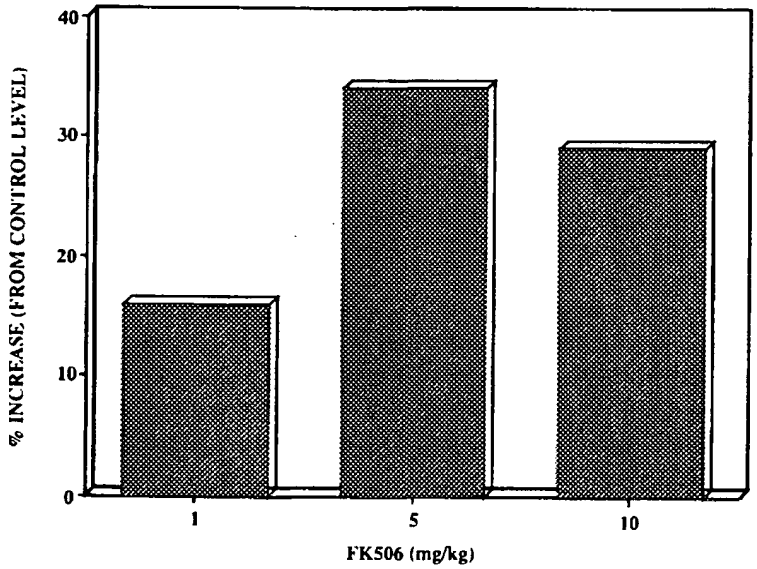

Fig. 4. FK506 dose-dependently increases the rate of axonal regeneration in the sciatic nerve. The percent increase in regeneration rate from control values increases with dose between 1 and 5 mg/kg, declining slightly in the 10 mg/kg group.

tion using radiolabeling techniques also exhibited a bell-shaped dose-response (Fig. 4); the percent increase in rate of axonal regeneration from control values was 16, 34, and 29% in the 1, 5, and 10 mg/kg groups, respectively. Taken together, these data establish the dose-dependency for the ability of FK506 to increase nerve regeneration in vivo.

Cyclosporin A Does Not Increase Axonal Regeneration Rate

To determine if calcineurin is involved in the ability of FK506 to increase the rate of axonal regeneration, we tested cyclosporin A to see if the drug shares FK506's regenerative properties (Wang et al., 1997). Since the drug–immunophilin complexes inhibit calcineurin activity to suppress T-cell function, it would be expected that equivalent doses of cyclosporin A would also increase the rate of axonal regeneration in a dose-dependent manner; i.e., if calcineurin inhibition is the underlying mechanism for FK506's nerve regenerative effect. Dosages (10 and 50 mg/kg/d) of cyclosporin A chosen for study were based upon its relative potency for immunosuppression and PPI (with cyclosporin A being approx 1/10 as potent as FK506) and our finding that a daily dose of 5 mg/kg of FK506 maximally increases the axon regeneration rate; the 50 mg/kg/d dosage approaches the maximal tolerated dose in rats and is sufficient to prevent allograft rejection in rodents (Cosenza et al., 1994). Neither dose of cyclosporin A altered the rate of axonal regeneration (as determined by radiolabeling techniques) nor did the drug speed functional recovery in the sciatic nerve. These findings indicate that cyclophilin A-mediated calcineurin inhibition does not mediate the ability of FK506 to increase nerve regeneration.

FKBP-12 Ligands Not Inhibiting Calcineurin Increase Nerve Regeneration

To determine whether FK506-FKBP-12 ligands increase nerve regeneration via a calcineurin-independent mechanism, studies have recently been conducted to test the regenerative potential of potent FKBP-12 inhibitors that do not inhibit calcineurin. Snyder and coworkers (Steiner et al., 1997) reported that *topical* administration of L-685,818 (18-OH, 21-ethyl-FK506) to the nerve-crush site accelerates functional recovery and increases axonal calibers (using paraffin-embedded nerves fixed by immersion in 10% formalin) distal to the crush lesion. Unfortunately, any correlation between functional recovery and morphological changes in these animals is problematic since the investigators limited their assessment of the axons to a distance of only 2 mm from the crush injury at 18 d following the lesion. It is therefore unclear whether the presence of more myelinated fibers is because of an effect of the drug on axonal sprouting as opposed to axonal elongation *per se*, an interpretation supported by their in vitro data (Steiner et al., 1997). Furthermore, the recent finding that L-685,818-FKBP-12 is able to inhibit calcineurin in *C. neoformans* (Odom et al., 1997) makes this compound of questionable value for definitively ruling out calcineurin activity in the nervous system.

Based upon the structural domains of FK506 (Fig. 5), two groups have synthesized novel small molecules that retain the FKBP-12 binding domain but *lack* the structural components of the effector domain. Snyder and coworkers reported (Steiner et al., 1997a,b) that *systemic* administration (i.e., subcutaneous injections for 18 d) of two FKBP-12 ligands (with binding affinities of 25- and 250-n*M*, respectively) but that do not inhibit calcineurin activity (and are not immunosuppressants) increase the size of myelinated fibers (at 2 mm distal to the crush site). As in the case of the L-685,818 compound, it is difficult to rule out an effect of the compounds on axonal sprouting, a possibility apparently supported by their findings in the striatum from *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice (Steiner et al., 1997b) (*see* FK506: Regeneration of CNS Neurons). This concern is exacerbated by their failure to assess functional recovery in animals given these compounds. These investigators also report an increase in myelination that may merely reflect the increase in axonal calibers, as opposed to a direct effect on myelination. Morphometric analysis of glutaraldehyde-fixed tissue is needed to differentiate between these two possibilities (e.g., calculating the G-ratio: diameter of the axon/diameter of the nerve fiber).

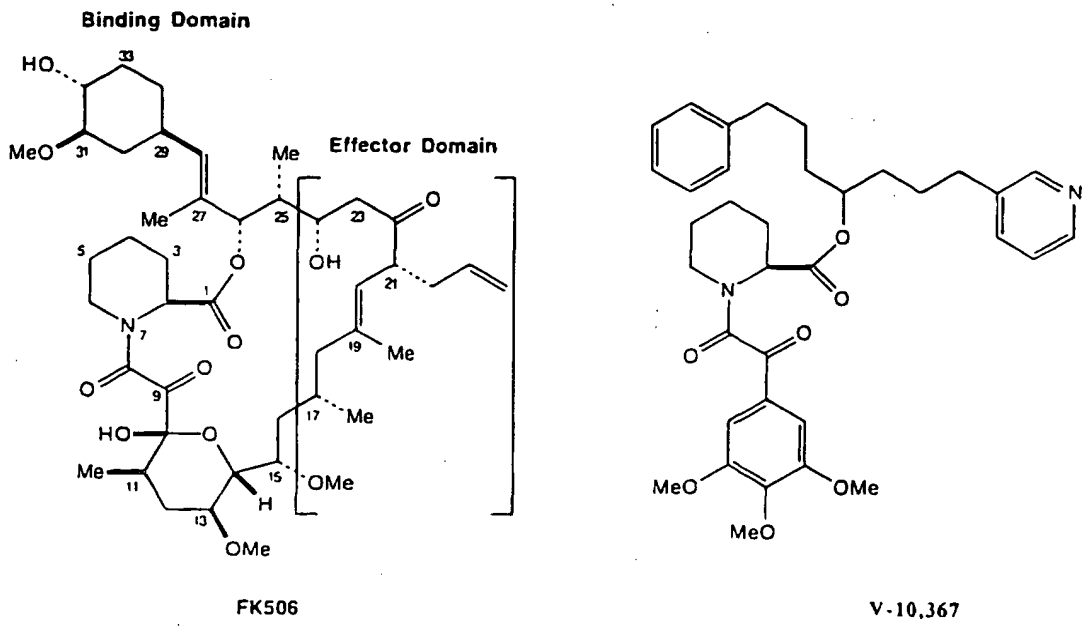

Fig. 5. Comparison of the structures of FK506 (left) and V-10,367 (right). The bracketed portion of FK506 represents the calcineurin-binding domain which is absent in V-10,367.

We have tested (Gold et al., 1997) by *systemic* administration (i.e., subcutaneous injections for 18 d), the nerve-regenerative property of V-10,367 (Fig. 5), a small molecule that also lacks the structural components of the effector domain necessary for calcineurin binding but that binds to FKBP-12 with much greater (<1 n*M*) affinity (Armistead et al., 1995) than the compounds used by Snyder and coworkers (Steiner et al., 1997a,b). As expected, this compound does not inhibit calcineurin activity (D. R. Armistead, personal communication). Rats given subcutaneous injections of V-10,367 (400 mg/kg/d) showed a more rapid (by approx 2 d) functional recovery following a focal sciatic nerve crush compared to vehicle-treated control animals. The morphological correlate of this earlier return of function was the presence of larger-sized regenerating axons in the sciatic nerve. By electron microscopy, mean axonal areas in the soleus nerve were 50% larger in the V-10,367-treated animals compared to control values. We found no evidence for an increase in myelination, as determined from the G-ratio (B.G. Gold and M.-S. Wang, unpublished observation). Whereas the increased size of regenerating axons was somewhat less than with FK506, further studies are needed to determine the optimal dosage of this compound. The compound is also effective by oral administration at dosages as low as 5 mg/kg (B. G. Gold, M. Zeleny-Pooley, and M.-S. Wang, unpublished observation). Thus, our study is the first to demonstrate that systemic administration of a potent FKBP-12 ligand that lacks the structural components necessary for calcineurin inhibition speeds functional recovery by accelerating the growth of regenerating axons to the distal musculature following a sciatic nerve-crush lesion. Future studies should address whether the compound also accelerates nerve regeneration in older animals as well as in other species.

FK506: Effects on Neurite Outgrowth in Culture

In the seminal 1994 study, Snyder and coworkers (Lyons et al., 1994) showed that FK506 potently (as low as 0.1 n*M*) increases

neuritic outgrowth from PC12 cells and DRG explant cultures in a concentration-dependent fashion. In PC12 cells, neurite outgrowth was assessed by determining the percentage of cells with processes greater than 5 μm. The ability of FK506 to enhance neurite outgrowth in PC12 cells was dependent upon the concentration of NGF. Maximal efficacy was found in the presence of relatively low (1–10 ng/mL) concentrations of NGF, since at higher concentrations of NGF (>10 ng/mL) the maximal response (in terms of % of cells bearing processes) was already demonstrated. Half-maximal response was obtained at 0.1 n*M* in the presence of submaximal (1 ng/mL) concentrations of NGF. The finding that similar results were obtained in DRG cultures indicates that FK506 activity is not dependent upon exogenously supplied neurotrophins (e.g., NGF) (*see* Putative Mechanisms for FK506's Ability to Increase Nerve Regeneration).

Recently, these investigators reported that cyclosporin A also increases neurite outgrowth in these two systems. We have used human neuroblastoma SH-SY5Y cells to study the concentration-dependency of FKBP-12 ligands for increasing neurite outgrowth. We measured neurite process length at 96 and 168 h after addition of the text agent. Maximal efficacy was observed between 1 and 10 n*M* in the presence of 10 ng/mL NGF. In contrast to Snyder and coworkers (Steiner et al., 1997a), and in accordance with our in vivo data (*see* Cyclosporin A Does Not Increase Axonal Regeneration Rate), we found that cyclosporin A (1–1000 n*M*) did not significantly alter neurite outgrowth in SH-SY5Y cells (B. G. Gold and M. Zeleny-Pooley, unpublished observations).

The results of these studies stand in contrast to those reported by Jay and coworkers (Chang et al., 1995) who found that FK506 *reduces* neurite outgrowth in cell culture. These studies employed a much higher (50 μ*M*) concentration of FK506 in the presence of very high levels of NGF (100 ng/mL). In this context, we have found that 1 μ*M* FK506 actually inhibits NGF-induced neurite outgrowth in SH-SY5Y cells (B. G. Gold and M. Zeleny-Pooley, unpublished results). Thus, in the context of the broad range of concentrations of FK506 we have examined, our results are, in fact, consistent with their findings.

FK506: Regeneration of CNS Neurons

Cell culture systems provide a simple means to examine whether FK506 also increases nerve regeneration in the CNS. We have conducted in vitro studies to examine whether FK506 also promotes axonal regeneration in cells not derived from the PNS. Our studies indicate that FK506 (1–100 n*M*), in the absence of exogenously supplied neurotrophins (e.g., neurotrophin-3; NT-3) also increased neuritic outgrowth in rat cortical neurons and hippocampal neurons (Fig. 6) in a concentration-dependent manner (B. G. Gold and M. Zeleny-Pooley, unpublished results).

An important question is whether FK506 is also effective in vivo in aiding nerve regeneration in the CNS. One preliminary report has examined the ability of daily subcutaneous injections of FK506 (0.5 mg/kg) to increase regeneration of dorsal-root axons into the spinal cord (Sugawara et al., 1995). This study was restricted to the examination of unmyelinated axons immunoreactive for calcitonin gene-related peptide (CGRP). Whether these axons originated in the DRG or represented abnormal sprouting of axons intrinsic to the spinal cord is unclear (Goldberger et al., 1993). Moreover, it is possible that in this model FK506 may have inhibited an autoimmune response leading to increased regeneration via its immunosuppression action.

A recent report (Steiner et al., 1997b) indicates that a nonimmunosuppressant FKBP-12 ligand also enhances the density of striatal dopaminergic innervation (as determined by immunocytochemical staining for tyrosine hydroxylase) in MPTP-intoxicated mice. Importantly, the FKBP-12 ligand increased the density of staining when given after the toxic agent,

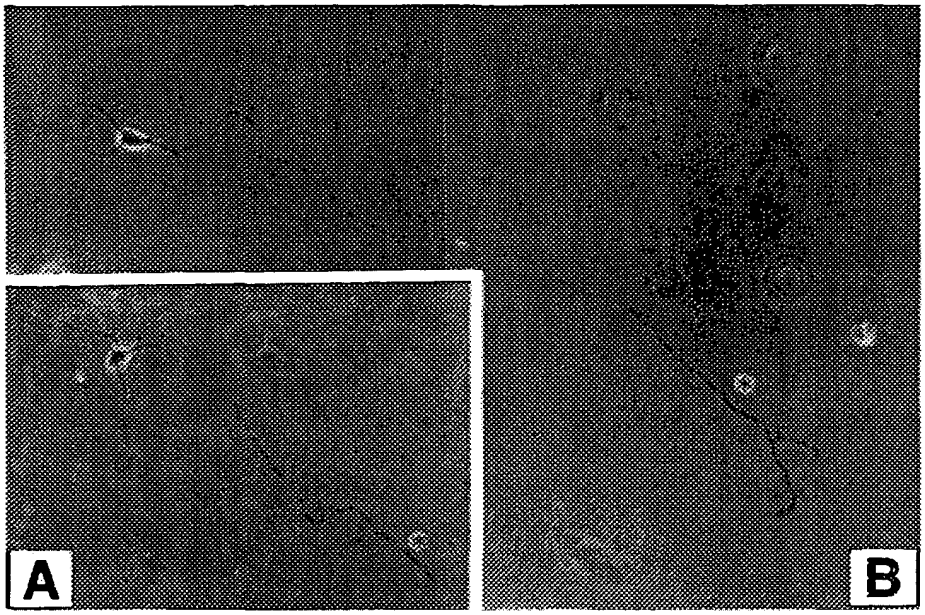

Fig. 6. Hippocampal neurons 86 h in culture either untreated **(A)** or treated with 100 n*M* FK506 **(B)**. The axonal processes (*) are clearly discernible and are markedly elongated in the neuron given FK506; neuritic length was 82 and 166 μm in **(A)** and **(B)**, respectively. Neurons were grown on coverslips that were inverted onto 24-well plates precoated with a monolayer of cortical astrocytes, according to Banker and Cowan (1977). Magnification 680X.

suggesting that the compounds can increase innervation following denervation. The increase in fiber density does not necessarily indicate regeneration of damaged fibers but may actually arise from collateral and/or terminal sprouting of intact axons. The alternative possibility that the increase in immunoreactivity merely reflects an increase in axonal transport of tyrosine hydroxylase cannot be ruled out. In addition, the possibility of inappropriate innervation of adjacent regions needs to be examined in detail. Whereas functional studies are needed to support the morphological data, a similar study in 6-hydroxydopamine-lesioned rats showed that the FKBP-12 ligand reduced motor disturbance (i.e., amphetamine-induced rotations). Further studies on the ability of FKBP-12 ligands to increase regeneration of CNS axons are warranted to assess their potential for the treatment of human neurodegenerative diseases.

FK506 Neuronal Actions: Calcineurin-Dependent and Calcineurin-Independent Mechanisms

Taken together, studies employing cyclosporin A and FK506-like compounds demonstrate that calcineurin inhibition is not necessary for FKBP-12 ligands to speed nerve regeneration and implicate a FKBP-pathway distinct from immunophilin-mediated inhibition of calcineurin in the ability of FK506 to increase axonal elongation. Thus, these findings do not support the hypothesis (Gold et al., 1995) that FK506 speeds axonal regeneration by increasing the phosphorylation state of GAP-43 secondary to calcineurin inhibition. However, it is possible that this pathway, by leading to a loss in the dynamics of GAP-43 phosphorylation (Meiri et al., 1991), could

negatively alter nerve regeneration, an explanation for the decreased drug efficacy observed at high (10 mg/kg) daily dosages (*see* FK506 Increases the Rate of Axonal Regeneration in the Rat Sciatic Nerve). Other calcineurin targets can also be discounted as playing a role in FK506's nerve regenerative effect. For example, inhibition of the calcineurin target NOS would lead to a reduction in NO formation (Dawson et al., 1993). Conflicting results have been reported on NO regulation of neurite outgrowth. A preliminary report using sensory neurons indicates that NOS inhibitors increase and, conversely, increased NO formation decreases neurite outgrowth in vitro (Wayne and Skene, 1995). In contrast, a recent paper (Hindley et al., 1997) shows that NO donors increase neurite outgrowth from PC12 cells and primary hippocampal neurons, a result contrary to that expected if NOS inhibition were to play a role in FK506's ability to increase nerve regeneration. Regardless of whether NO positively or negatively impacts neurite outgrowth, a role for the calcineurin target NOS in nerve regeneration is ruled out by studies using FKBP-12 ligands lacking calcineurin inhibition (Steiner et al., 1997a,b; Gold et al., 1997). However, it should be noted that calcineurin inhibition may play a role in mediating other important neuronal properties of FK506. For example, FK506's neuroprotective action against glutamate toxicity in vitro may utilize this pathway (Dawson et al., 1993; Snyder and Sabatini, 1995). In addition, the demonstration that systemic injections of FK506 (Kitamura et al., 1994) and cyclosporin A (Matsuura et al., 1996) protect striatal neurons against depletion of dopamine by MPTP or 6-hydroxydopamine, respectively, strongly suggests that calcineurin inhibition may also mediate this neuroprotective action. Together with the finding that a FKBP-12 ligand increases regeneration of dopaminergic neurons in MPTP-treated mice, these exciting findings suggest that FK506 (or related compounds) may be beneficial in the treatment of Parkinson's disease.

Putative Mechanisms for FK506's Ability to Increase Nerve Regeneration

The mechanism by which FK506 increases the rate of peripheral nerve regeneration is unknown. Whereas our studies (Wang et al., in press) (*see* FKBP-12 Ligand Not Inhibiting Calcineurin Increases Nerve Regeneration) appear to rule out a calcineurin-dependent mechanism, it is possible that FKBP-12 may still be involved, albeit via a different pathway. For example, the FKBP-12 has also been found to demonstrate stable interactions with the two Ca^{2+} channels release Ca^{2+} from internal stores: the ryanodine (Timerman et al., 1993; Brillantes et al., 1994; Giannini et al., 1995) and the inositol 1,4,5-triphosphate (IP_3) receptors (Cameron et al., 1995a,b); FKBP and calcineurin interact under physiological conditions to modulate Ca^{2+} flux in these channels and FK506, by inhibiting calcineurin and preventing receptor dephosphorylation, increases Ca^{2+} transport through these channels. The possibility that FK506's ability to stabilize these channels and alter Ca^{2+} release (Brillantes et al., 1994; Cameron et al., 1995b) is involved in the drug's regenerative effects (*see* Snyder and Sabatini, 1995) appears to be supported by a very recent preliminary report (Takei et al., 1996) showing that inactivation of the type 1 IP_3 receptor in chick DRG growth cones inhibits neuritic growth. However, the somewhat higher concentrations of FK506 (10–100 n*M*) necessary to disrupt association of FKBP-12 from the IP_3 receptor (Cameron et al., 1995a), compared to concentrations that enhance neurite outgrowth in vitro (Lyons et al., 1994; B. G. Gold and M. Zeleny-Pooley, unpublished observation), makes this possibility less attractive.

An alternative mechanism by which FK506 could alter nerve regeneration via FKBP-12 is suggested by the finding that FKBP-12 is a natural ligand for the type 1 receptor for transforming growth factor-β (TGF-β1), where it functions as an inhibitor of TGF-β1 receptors (Wang et al., 1994, 1996). Since TGF-β1 stimulates nerve

growth factor (NGF) synthesis by glial cells (Lindholm et al., 1990), and NGF has been suggested to play a role in axonal elongation (Taniuchi et al., 1988), FK506 could increase regeneration indirectly via an increase in NGF. Such a mechanism may be supported by the findings that FK506 appears to increase the sensitivity of PC-12 cells to NGF (Lyons et al., 1994) and by the demonstration that TGF-β1, at similarly low concentrations (i.e., 1 ng/mL), promotes regrowth of injured neurites in vitro (Abe et al., 1996). However, this hypothesis is unattractive given that a role for NGF in axonal regeneration is not supported by in vivo studies (Diamond et al., 1992); in fact, we have shown recently that delivery of NGF to the neuronal cell body (by intrathecal infusion) delays regeneration (Gold, 1997). Furthermore, FKBP-12 has been shown to be dispensable for TGF-β signaling (Charng et al., 1996). In addition, as in the case of the IP_3 receptor (*see* preceding paragraph), much higher concentrations of FK506 (μ*M*) are needed to disrupt association of FKBP-12 from the TGF-β1 receptor (Wang et al., 1994) relative to those that stimulate neurite outgrowth in vitro (Lyons et al., 1994); B. G. Gold and M. Zeleny-Pooley, unpublished observation.

An unexplored area is the possible involvement of FKBPs besides FKBP-12 in mediating FK506's nerve regenerative effect. Most interesting is the FK506–FKBP-52 complex, which does not inhibit calcineurin (*see* Snyder and Sabatini, 1995). Instead, human FKBP-52 (rabbit FKBP-59) (human FKBP-52) (Tai et al., 1992) has been identified as a heat-shock protein (hsp-56) (Sanchez, 1990) and, together with the hsp-90 and hsp-70, this novel immunophilin comprises a component of a subclass of glucocorticoid receptor complexes (Owens-Grillo, et al., 1995; Perdew and Whitelaw, 1991; Lebeau et al., 1992; Czar et al., 1994); FK506 does not alter glucocorticoid signaling pathways but may produce conformational changes in the complex (Ratajczak and Carrello, 1996). In this context, we have found that subcutaneous injection of FK506 (10 mg/kg/d, for 2 wk) increases expression of hsp-70 (as shown by immunocytochemistry) in selected neurons in the brain (including the cortex, hippocampus, and amygdala), and in the spinal cord and dorsal root ganglion (DRG) (Goto and Singer, 1994). It may therefore be relevant that the glucocorticoid dexamethasone has been found to increase GAP-43 mRNA levels in regenerating hypoglossal neurons (Yao and Kiyama, 1995). Moreover, it has recently been shown that FK506 potentiates the potency of steroid hormones (Kralli and Yamamoto, 1996). Thus, our (B. G. Gold, J. Y. Yew, and M. Zeleny-Pooley, unpublished observations) recent demonstration that FK506 increases GAP-43 mRNA levels in DRG neurons may support an involvement of this pathway in FK506's ability to increase nerve regeneration.

Consistent with our nerve regenerative studies (*see* Cyclosporin A Does Not Increase Axonal Regeneration Rate), the ability of FK506 to protect hippocampal CA1 and cerebral neurons against transient global ischemia is not shared by cyclosporin A (Yagita et al., 1996). This suggests that a calcineurin-independent mechanism also underlies FK506's ability to provide neuroprotection against ischemic injury. The recent demonstration that global ischemia induces an alteration in the ryanodine receptor (Nozaki et al., 1996) suggests that FK506 via the FKBP-12 may protect against damage via binding to the FKBP-12. However, the necessity of using repeated drug administration (Drake et al., 1996) suggests that simple inhibition of NO may not fully explain the neuroprotective actions of FK506. FK506's ability to protect against focal cerebral ischemia (Sharkey and Butcher, 1994; Ide et al., 1996; Tokime et al., 1996) may therefore involve additional FKBP pathways, perhaps mediated by hsp-70 induction via glucocorticoid receptors. Whether this pathway also plays a role in the ability of FK506 to protect against ischemic damage is an important area for future work.

Why FK506 exhibits a reduction in efficacy for axonal regeneration at high (10 mg/kg) dosages is unknown. Interestingly, a bell-shaped dose-response has been observed for other agents that increase regeneration. For example, the melanocortins (α-MSH), insulin-like growth factor-I, and ACTH demonstrate a

similar decrease in efficacy beyond a certain optimal dosage (Bijlsma et al., 1981; Contreras et al., 1995; Bijlsma et al., 1983); however, for the latter two agents, hypoglycemia and corticosteroid activity, respectively, may be responsible for the reduced regenerative efficacy of high dosages (Bijlsma et al., 1981; Contreras et al., 1995). For the melanocortins, it has been suggested (Joosten et al., 1996) that the bell-shaped dose-response is caused by the action of multiple melanocortin receptors (Hol et al., 1995). Whereas alternative mechanisms are indeed possible, the reduced efficacy of higher (10 mg/kg) dosages of FK506 suggests that multiple FKBPs (Snyder and Sabatini, 1995; Sigal and Dumont, 1992; Cardenas et al., 1995; Wiederrecht et al., 1993) or FKBP-mediated pathways (acting in opposition) underlie the ability of FK506 to alter nerve regeneration. For example, whereas the results presented in this review indicate that FK506 speeds regeneration via a calcineurin-independent pathway, calcineurin inhibition (which may be less sensitive) would be expected to impair regeneration (Chang et al., 1995) leading to a reduction in efficacy at higher (10 mg/kg) dosages of FK506.

Summary and Future Directions

Taken together, it appears that distinct mechanisms underlie the immunosuppressant (calcineurin-dependent) and nerve regenerative (calcineurin-independent) properties of FK506. Based upon structural analysis of FK506-FKBP interactions (Griffith et al., 1995; Itoh et al., 1995a,b), it has been possible to separate these properties and design new FKBP ligands (Armistead et al., 1995; Batchelor et al., 1994; Shuker et al., 1996) that do not inhibit calcineurin. Of equal importance for drug development is the possibility that different pathways underlie FK506's nerve regenerative (Wang et al., 1997; Steiner et al., 1997a,b; Gold et al., 1997) and neurotoxic properties (Lopez et al., 1991; Mueller et al., 1994; Wijdicks et al., 1995; Bronster et al., 1995; Vincenti et al., 1996). The latter have been linked mechanistically to the immunosuppressant properties of FK506 via calcineurin inhibition (Dumont et al., 1992); calcineurin inhibition also has been shown to underlie development of some pathological hallmarks of Alzheimer's disease (Ladner et al., 1996). Thus, it is possible that FK506's neurotoxic properties will not be shared by these new (noncalcineurin-binding) FKBP ligands.

It should be apparent, however, that our knowledge is critically lacking in regard to the underlying molecular mechanism(s) by which FKBP ligands produce their neuronal effects. Of paramount importance will be identification of the molecular targets for FKBP ligand-FKBP complexes. This information should become available in the near future given the recent development of innovative new techniques such as the three-hybrid system (Licitra and Liu, 1996) to "fish" for such targets. It will then be necessary to sort out which of these targets are physiologically relevant for promoting nerve regeneration. These studies should also shed new light on the neuron's intrinsic mechanism(s) regulating axonal elongation. Regardless of the underlying mechanism involved, the development of novel FKBP ligands (Navia and Chaturvedi, 1996) may lead to the generation of new drugs for the treatment of human nerve injuries.

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Specificity, diversity, and regulation in TGF-β superfamily signaling

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ABSTRACT Transforming growth factor-β (TGF-β) superfamily members are multifunctional cell–cell signaling proteins that play pivotal roles in tissue homeostasis and development of multicellular animals. They mediate their pleiotropic effects from membrane to nucleus through distinct combinations of type I and type II serine/threonine kinase receptors and their downstream effectors, known as Smad proteins. Certain Smads, termed receptor-regulated Smads, become phosphorylated by activated type I receptors and form heteromeric complexes with a common-partner Smad4, which translocates into the nucleus to control gene transcription. In addition to these signal transducing Smads, inhibitory Smads have been identified that inhibit the activation of receptor-regulated Smads. In contrast to the still growing TGF-β superfamily (with ~30 members in mammals), relatively few type I and type II receptors as well as Smads have been identified. We will focus on recent insights into the molecular mechanisms by which signaling specificity between different TGF-β superfamily members is achieved and regulated, and how a single family member can elicit a broad scala of biological responses.—Piek, E., Heldin, C.-H., ten Dijke, P. Specificity, diversity, and regulation in TGF-β superfamily signaling. *FASEB J.* 13, 2105–2124 (1999)

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THE TGF-β SUPERFAMILY AND ITS BINDING PROTEINS

TGF-β superfamily: multiple factors with pleiotropic functions

THE TRANSFORMING GROWTH FACTOR-β (TGF-β) SUPERFAMILY is composed of many multifunctional cytokines including TGF-βs, activins, inhibins, anti-müllerian hormone (AMH), bone morphogenetic proteins (BMPs), myostatin, and others. The highly similar TGF-β isoforms TGF-β1, TGF-β2, and TGF-β3 potently inhibit cellular proliferation of many cell types, including those from epithelial origin. Most mesenchymal cells, however, are stimulated in their growth by TGF-β. In addition, TGF-βs strongly induce extracellular matrix synthesis and integrin expression, and modulate immune responses (reviewed in refs 1, 2). BMPs are potent inducers of bone and cartilage formation and play important developmental roles in the induction of ventral mesoderm, differentiation of neural tissue, and organogenesis (reviewed in refs 3, 4). Activins, named after their initial identification as activators of follicle-stimulating hormone (FSH) secretion from pituitary glands, are also known to promote erythropoiesis, mediate dorsal mesoderm induction, and contribute to survival of nerve cells (reviewed in ref 5). Several growth factors belonging to the TGF-β superfamily play important roles in embryonic patterning and tissue homeostasis. Their inappropriate functioning has been implicated in several pathological situations like fibrosis, rheumatoid arthritis, and carcinogenesis.

Distinct *in vivo* expression patterns of TGF-β isoforms

TGF-β1, TGF-β2, and TGF-β3 are highly similar in their biological activities *in vitro*. However, they differ in their *in vivo* expression patterns (reviewed in ref 6), which largely explains the unique isoform-specific phenotypes displayed by the TGF-β knockout mice. TGF-β1-deficient mice that are born alive undergo early postnatal death due to excessive infiltration of inflammatory lymphocytes and macrophages into several organs (7–9). Half of the mice lacking TGF-β1 die *in utero* due to defects in vasculogenesis and hematopoiesis (10). It is thought that maternally supplied TGF-β1, in combination with redundant expression of TGF-β isoforms, contributes to normal development of the embryos. TGF-β1 null mice born from TGF-β1-deficient mothers display abnormal cardiac development (11). TGF-β2 knockout mice show multiple developmental malformations of tissues and organs, leading to perinatal death (12). TGF-β3 null mice die shortly after birth, displaying delayed pulmonary development and defective palate development (13, 14).

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Analysis of the genes encoding the three TGF-βs revealed that each isoform is c ntrolled by differentially regulated promoters (reviewed in ref 15). The existence of different genes encoding functionally similar proteins, yet controlled by differentially regulated promoters, might provide an important mechanism to ensure tissue-specific and spatio-temporal expression patterns of the different TGF-β isoforms, thereby resulting in proper embryonic development and tissue homeostasis.

In addition, posttranscriptional control mechanisms contribute to regulation of the production of TGF-β. TGF-β1, TGF-β2, and TGF-β3 that have long, GC-rich 5′-untranslated regions and intramolecular duplex loops located in close proximity to the transcriptional start site negatively modulate TGF-β expression, possibly by binding cell type-specific cytoplasmic molecules (16).

C ntrol of TGF-β bioactivity

Members of the TGF-β superfamily are synthesized as large precursor molecules that are proteolytically processed in the Golgi apparatus by the convertase family of endoproteases, of which furin is a member. Furin cleaves the precursor into a mature TGF-β and amino-terminal precursor remnant, also termed latency-associated protein, or LAP (reviewed in ref 17 and references therein; 18). LAP remains noncovalently linked to TGF-β and prevents binding of mature TGF-β to its receptors. These so-called small latent TGF-β complexes are significantly more stable than bioactive TGF-β. Within the Golgi, LAP covalently interacts with latent TGF-β binding proteins (LTBPs), to form large, latent complexes (reviewed in ref 19 and references therein). Four different LTBP homologs, LTBPs 1–4, have been characterized, including several alternative splice variants (reviewed in refs 19, 20). LTBPs function to enhance secretion and stability of the TGF-β-LAP complex, ensure correct folding of TGF-β, and target the latent TGF-β complex to the extracellular matrix of certain cells and tissues for storage or to the cell surface where activation takes place (reviewed in refs 17, 19 and references therein). LTBP-1, -2, and -4 contain RGD sequences that are integrin binding sites (20, 21). It has been shown that large latent TGF-β complexes directly interact with integrin α_v/β_1 at the cell surface (21), which may enable TGF-β to activate integrin signaling. Activation of latent TGF-β into biologically active mature TGF-β is controlled by proteases like plasmin or cathepsin, which cleave LAP (**Fig. 1**), and by binding of LAP to mannose-6-phosphate receptors (17, 19 and references therein). Lipoprotein Lp(a) inhibits activation of plasminogen to plasmin and thereby negatively affects activation of TGF-β (reviewed in ref 22 and references therein). Other mechanisms that have been implicated in activation of latent TGF-β are deglycosylation of LAP (23), exposure to reactive oxygen species (24), or acidic cellular microenvironments (25). In addition, an important activator of TGF-β *in vivo* appears to be thrombospondin-1, which induces a conformational change of LAP and thereby activates TGF-β (26).

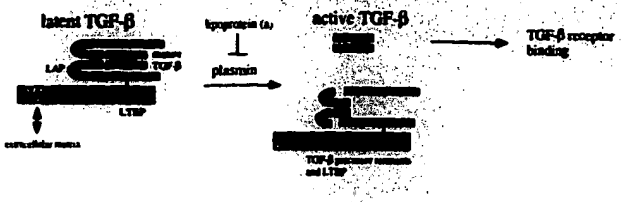

Figure 1. Activation of latent TGF-β. TGF-β is synthesized as large latent complexes. In platelets the TGF-β latent complex consists of mature TGF-β that is noncovalently associated with a disulfide-bonded complex of a dimer of the amino-terminal propeptide of the TGF-β precursor (also termed latency-associated peptide, LAP) and a third component, termed laten: TGF-β binding protein (LTBP). Active TGF-β (capable of receptor binding) can be released from the latent complex by specific proteases, like plasmin. This process is likely to occur at the plasma membrane. Lipoprotein Lp(a) is structurally related to plasminogen, the precursor of plasmin. Lp(a) can inhibit plasmin generation, and inhibit activation of latent TGF-β. An alternative mechanism for activation of latent TGF-β is through thrombospondin, which appears to induce a conformational change in LAP (not shown).

Signaling diversity by generation of heterodimeric ligands

After proteolytic cleavage of the mature carboxyl-terminal parts, biologically active TGF-β1, -β2, or -β3 homodimers are generated. Homodimers are most abundant, but TGF-β1.2 (27, 28) and TGF-β2.3 (28) heterodimers have been identified *in vivo*. For activin, four different β chains have been identified—βA, βB, βC, and βE (reviewed in ref 29 and references therein)—of which βA and βB are known to form homo- as well as heterodimers. Whether heterodimers can also be formed with the βC and βE chains and whether these different isoforms exert different biological activities is not clear. Inhibins, which are heterodimers of inhibin α chains and activin β chains, are functional antagonists of activin signaling (reviewed in ref 5). In the case of BMPs, BMP2/7 and BMP4/7 heterodimers are much more potent in the induction of ventral mesoderm (30), as well as in bone induction (31) than their respective homodimers. The coexpression of individual BMPs in several tissues suggests that heterodimer formation might occur *in vivo*, and heterodimers of BMP2 and BMP-7 have been isolated from bovine bone (32). The mechanism underlying potentiated signaling by heterodimers compared to homodimers has not been investigated, but might be due to formation

TABLE 1. *TGF-β superfamily binding proteins*

TGF-β superfamily binding protein	Interacting ligands	Molecular characteristics	Functional properties	Reference
Decorin	TGF-βs	Extracellular, proteoglycan	Storage in extracellular matrix, inhibitor of TGF-β activity	33
Biglycan	TGF-βs	Extracellular, proteoglycan	Storage in extracellular matrix, inhibitor of TGF-β activity	33, 34
60 kDa protein	TGF-βs	Extracellular, matrix-associated	Inhibitor of ligand-receptor interaction	62
α2-Macroglobulin	TGF-βs, activins	Serum component	Clearance factor	35, 36
Follistatin	Activins, BMPs	Extracellular, heparan-sulfate proteoglycan binding protein	Inhibitor of ligand-receptor interaction	Reviewed in ref 36
Noggin	BMPs	Extracellular, soluble	Inhibitor of ligand-receptor interaction	Reviewed in ref 40
Chordin	BMPs	Extracellular, soluble	Inhibitor of ligand-receptor interaction	Reviewed in ref 40
Cerberus	BMPs, activins	Extracellular, soluble	Inhibitor of ligand-receptor interaction	42
Dan	BMPs	Extracellular, soluble	Inhibitor of ligand-receptor interaction	42
Gremlin	BMPs	Extracellular, soluble	Inhibitor of ligand-receptor interaction	42
TβR-III	TGF-βs	Transmembrane, proteoglycan	Accessory receptor; ligand presentation	46, 49, 51
Endoglin	TGF-β1, TGF-β3, activins, BMPs	Transmembrane, proteoglycan	Unknown, signal modulator?	47, 53
Xnr3	BMPs	Intracellular, nodal-related	Inhibitor of ligand synthesis	Reviewed in ref 40

of receptor complexes consisting of, for example, two different type II and two different type I receptors, which may activate downstream signaling in a more potent manner.

TGF-β superfamily binding proteins

After production of bioactive TGF-β superfamily members, several extracellular proteins can bind and modify their activity (**Table 1**). The extracellular matrix proteoglycans decorin and biglycan have been described to inhibit TGF-β activity (33). Transgenic mice lacking biglycan expression show dramatically decreased bone mass, suggesting an important role for biglycan in the constitution of bone, possibly by its function as a source for storage of TGF-β superfamily members (34).

The serum protein α2-macroglobulin associates with circulating TGF-β superfamily members; via its interaction with the α2-macroglobulin receptor, it takes care of clearance of the growth factors from serum (35, 36). Fibronectin can bind TGF-β and promote its bioactivity, possibly by changing it from a latent to a bioactive conformation (37), and collagen IV may store bioactive TGF-β in the extracellular matrix (38).

Factors known to play important roles during embryonic development are follistatin, noggin, chordin, and Short gastrulation (Sog), the *Drosophila* homologue of chordin (39). These proteins are secreted by the Spemann organizer and compete with BMPs to exert opposing effects on developmental fates of mesoderm and ectoderm (reviewed in ref 40 and references therein). Mice deficient in noggin expression display excessive cartilage formation and failure of joint specification during skeletogenesis (41). The TGF-β superfamily member Xnr3 has been reported to interfere with BMP signaling in the *Xenopus* Spemann organizer via an intracellular antagonistic mechanism that prevents BMP synthesis (reviewed in ref 40).

Cerberus, DAN, and gremlin have recently been identified as antagonists of BMP signaling during

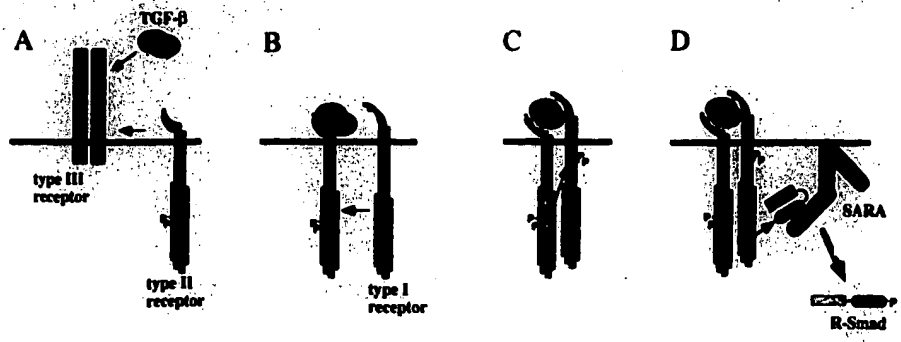

Figure 2. Mechanism of TGF-β receptor activation. Schematic stepwise illustration of the current model for TGF-β receptor activation. *A)* TGF-β1 initially binds the accessory TGF-β type III receptor (TβR-III), which presents the ligand to TGF-β type II receptor (TβR-II). *B)* Subsequently, TGF-β bound to TβR-II, recruits TGF-β type I receptor (TβR-I) into the complex, thereby forming a heteromeric complex of two TβR-IIs and two TβR-Is. *C)* The constitutively active TβR-II kinase phosphorylates TβR-I. *D)* TβR-I propagates the signal downstream through phosphorylation of particular R-Smads, i.e., Smad2 and Smad3. Recruitment of Smad2 and Smad3 to the TGF-β receptor complex is achieved through the Smad anchor for activation (SARA). SARA is membrane associated and capable of binding both R-Smad and the TGF-β receptor complex.

early embryogenesis (42). Cerberus can also counteract signaling by activin and nodal. These proteins, like noggin and chordin, have been shown to interact directly with the growth factors, thereby preventing interaction of the ligands with their signaling receptors (reviewed in refs 40, 42). Follistatin counteracts activin signaling by a similar mechanism (reviewed in ref 36), whereas the mechanism by which follistatin interferes with BMP signaling is unclear; BMP–follistatin complexes were found to interact normally with BMP receptors (43).

Interaction of chordin or Sog with BMPs is controlled by metalloproteases of the astacin family, to which tolloid, the *Drosophila* homologue of BMP1, *Xenopus* xolloid, and zebrafish Ztld, belongs, and they are able to proteolytically cleave chordin and Sog, thereby liberating bioactive BMPs (44; reviewed in ref 45).

An extensive number of membrane-bound or transmembrane TGF-β binding proteins are known to interfere with TGF-β action. The type I and type II serine/threonine kinase receptors, which will be discussed in detail below, are directly involved in TGF-β signal transduction. TGF-β receptor III (TβR-III, also known as betaglycan) and endoglin are transmembrane proteins that share high sequence homology in their short cytoplasmic tails rich in serine residues (46, 47). Betaglycan has several high-affinity binding sites for TGF-β1, TGF-β2, and TGF-β3 (48) and facilitates binding of TGF-β to their type II receptors, a property that is especially important for TGF-β2, which has low intrinsic affinity for TβR-II (**Fig. 2**) (49, 50). In contrast, soluble betaglycan acts as an antagonist of TGF-β bioactivity by preventing the interaction of TGF-β with the signaling receptors (46, 51).

The role of endoglin in modulation of TGF-β signaling appears to be different from betaglycan. In contrast to betaglycan, endoglin can interact with TGF-β1, TGF-β3, activin A, BMP-2, and BMP-7 and requires appropriate type I or type II receptors for efficient ligand binding (52). Ectopic expression of endoglin in monocytes results in a selective abrogation of TGF-β1-induced growth inhibition and fibronectin synthesis (53). Furthermore, mutations in the genes encoding endoglin or ALK-1, a putative TGF-β type I receptor in endothelial cells, form the basis for the vascular disorder hereditary hemorrhagic telangiectasia type 1 and type 2, respectively (54, 55), suggesting that endoglin and ALK-1 act in a common signal transduction pathway.

In many vascular endothelial cells and hematopoietic progenitor cells, TGF-β1 and TGF-β3 are equipotent in mediating signaling, but TGF-β2 has weaker biological effects (47, 56). This difference in potency between different TGF-β isoforms can be accounted for by the lack of betaglycan in these cells. Differential activities of TGF-β isoforms in other cell types have been observed, but their modes of action have not been correlated to receptor binding properties (57, 58). Several cell membrane associated TGF-β binding proteins, including certain glycosyl-phosphatidylinositol (GPI)-anchored proteins (59, 60), have been identified that are endowed with specific affinity for certain TGF-β isoforms, but their role in TGF-β signaling is not clear. Furthermore, a 60 kDa TGF-β binding protein has been characterized (61, 62) that exhibits TGF-β1 binding specificity and interferes with binding of TGF-β to the signaling receptors (62).

SIGNALING VIA TGF-β SUPERFAMILY RECEPTORS

TGF-β superfamily members induce formation of h teromeric complexes of type I and typ II recept rs

Type I and type II serine/threonine kinase receptors are directly involved in signaling by TGF-β superfam-

ily members (Fig. 2). TGF-β1, TGF-β3, and activins initially bind to their corresponding type II receptors, after which the type I receptors are recruited into the signaling complex. Type I and type II receptors have an intrinsic affinity for each other, which contributes to stability of the heteromeric complex (63). TGF-β2, which does not bind to TβR-II or TβR-I alone, can interact with and stabilize transiently formed complexes of TβR-I and TβR-II (64). BMPs have low affinity for type II or type I receptors individually, and high-affinity binding requires formation of a heteromeric type I/type II receptor complex (65, 66). At present, only a few type I and type II receptors for the TGF-β superfamily are known. However, depending on the ligand, multiple heterotetrameric complexes consisting of two type I and two type II receptor homodimers are possible (67). In view of the existence of heterodimeric ligands, it is conceivable that two different type I and two different type II receptors constitute the receptor complex, thereby creating combinatorial signaling.

TGF-βs form heteromeric complexes between TβR-II and TβR-I in most cell types (68, 69). In endothelial cells TGF-β can bind and signal through activin receptor-like kinase 1 (ALK-1); ALK-1 has therefore been implicated as an endothelial-specific TGF-β type I receptor (T. Imamura, P. ten Dijke, and K. Miyazono, personal communication). Activins signal via combinations of ActR-II or ActR-IIB and ActR-IB (68, 70). Activins have also been shown to interact with ActR-I (68, 71), although this receptor appears to play a minor role in actuvin signaling. BMPs interact with ActR-II, ActR-IIB, or BMPR-II, in combination with ActR-I, BMPR-IA, or BMPR-IB (65, 66, 72). For AMH, only a type II receptor has been identified (73). A potential AMH type I receptor is ActR-I, which is coexpressed with AMHR-II in the Müllerian duct and can mediate AMH-specific repression of FSH-induced aromatase activity (A. Themmen, personal communication). ALK-7 is an orphan receptor (74). A novel type I receptor, designated zALK-8, was recently cloned and shown to be widely expressed throughout early zebrafish development (75). Mammalian homologues of zALK-8 as well as of zebrafish TARAM-A, which is a type I receptor involved in induction of anterior dorsal mesoderm (76), have not yet been identified. The ligands for these receptors have not been elucidated.

Activation and regulation of serine/threonine kinase typ I and type II recept rs

Type I receptor kinases become activated upon phosphorylation by the constitutively active type II receptor kinase (Fig. 2). Thus, type I receptors act downstream of type II receptors (77). Consistent with this finding, type I receptors were found to determine the specificity of the intracellular signals induced by different TGF-β superfamily members, whereas the L45 region in type I receptors was found to be important in determining the specificity of type I receptors (78–80).

The phosphorylation status of TβR-II has been shown to influence TGF-β signaling. TβR-II is a constitutively active kinase that requires phosphorylation of Ser213 (located outside the kinase domain) to mediate its autocatalytic effect. Ligand-dependent autophosphorylation of TβR-II on either Ser409 or Ser416 differentially contributes to regulation of its activity, leading to stimulation or inhibition of TGF-β signaling, respectively (81).

Differential kinetics in biosynthesis, ligand-induced internalization, and down-regulation of type I vs. type II receptors have been attributed to differential modulation of TGF-β signaling (82–85). The half-life and processing of TβR-II in the endoplasmic reticulum are considerably shorter than that of TβR-I (82–85). Furthermore, homo- and heteromeric receptor complexes have distinct endocytotic fates (84). Rapid modulation of TβR-II expression levels as well as ligand-bound TβR-I/TβR-II complexes may be important for fine-tuning of signaling by TGF-β.

Diversity in receptor availability might be provided by the existence of alternative receptor transcripts, as identified for ActR-IIA (86), ActR-IB (87), ActR-IIB (70), AMHR-II (73), and TβR-II (88). In the case of ActR-IIB, differential affinity of activin for the alternatively spliced gene products has been observed. In addition, differences in the cytoplasmic domains offer possibilities for differential modes of signaling (70).

Several growth factors are known to bind to the same type II receptor—for example, activins and BMPs, which share ActR-II and ActR-IIB (72, 89). This enables signaling by several growth factors via a limited scala of available receptor types, whereas it can lead to competition of different ligands for the same type II receptors, thereby fine-tuning signaling via type I receptors. This is the case for activin and inhibin, since inhibin is known to counteract many of activin functions by competing for binding to the activin type II receptor (90). A similar mechanism has been suggested to underlie the inhibitory action of zebrafish antivin, most closely related to mouse lefty, upon activin signaling (91), and appears to contribute to the functional antagonism observed between activin and OP-1 in Tera-2 embryonal carcinoma cells (89). Whether different ligands might also compete for binding to common type I receptors is not clear at present.

TABLE 2. *TGF-β superfamily receptor interacting proteins*

Receptor-interacting protein	Interacting receptor type	Molecular characteristics	Functional properties	Reference
FKBP12	Type I receptors	Immunophilin	Inhibitor of TβR-I kinase	97, 98
FTα	TGF-β type I receptor	Farnesyl transferase	Unknown	99
TRIP-1	TGF-β type II receptor	WD-40 repeat protein	Signal modulator	92, 93
STRAP	TGF-β type I, II receptors	WD-40 repeat protein	Inhibitor of transcriptional responses	94
TRAP-1	TGF-β type I receptor	Without motif	Inhibitor of TGF-β signaling	96
Bα	TGF-β type I receptor, activin type I receptor	WD-40 repeat protein, phosphatase 2A-subunit	Enhancer of growth inhibition	95
Apolipoprotein J	TGF-β type I, II receptors	Nuclear protein or secreted glycoprotein	Role in TGF-β receptor signaling and/or processing	227
SARA	TGF-β receptor complex	FYVE-containing protein	Smad subcellular localizer	147
Smad	Type I receptors	Transcription factor	Signal transducer	29, 101
BRAM-1	BMP type IA receptor	E1A-associated protein	Unknown	212
XIAP	BMP type IA receptor	Inhibitor of apoptosis protein	Adaptor protein for TAB1, inhibitor of apoptosis	211

Cytoplasmic TGF-β receptor interacting proteins distinct from Smads

A number of cytoplasmic proteins are now known to interact with the kinase domain of type I and type II receptors, thereby manipulating or mediating their signaling capacities (Table 2). TRIP-1, a protein that contains five WD domains, is known to bind and become phosphorylated by TβR-II. The interaction requires a functional TβR-II kinase (92). Overexpression of TRIP-1 attenuates TGF-β-induced PAI-1 transcriptional response, but not TGF-β-induced cyclin A response (93). The WD domain-containing protein STRAP can associate with TβR-I and TβR-II. STRAP and the inhibitory Smad7 synergistically block TGF-β-mediated transcriptional activation (94). Another WD-40 repeat protein Bα, a subunit of protein phosphatase 2A, has been reported to interact with and become phosphorylated by TβR-I. Bα potentiates the antiproliferative effect mediated by TGF-β (95). TβR-I-associated protein-1 (TRAP-1) interacts specifically with the activated TβR-I. However, its functional role in TGF-β signaling is unclear (96).

The immunophilin FKBP12 interacts with type I serine/threonine kinase receptors (97) through an FK506-like Leu-Pro motif preceding the kinase domain (98). FKBP-12 inhibits phosphorylation of the type I receptors by the type II receptors, possibly by sterical hindrance, and may function to prevent leaky signaling in the basal cell state (99). FKBP12 dissociates from TβR-I after ligand-induced phosphorylation of TβR-I by TβR-II (97, 98).

Farnesyltransferase α (FTα) has also been shown to interact with and become phosphorylated by activin and TGF-β type I receptors (99). However, farnesyl transferase activity was shown to be dispensable for TGF-β signaling (100).

Type I receptors as determinants of downstream signal propagation

Activation of type I receptors by type II receptors occurs by phosphorylation of serine and threonine residues within a juxtamembrane region preceding the kinase domain of type I receptors that is characterized by repeats of glycine and serine residues (also termed the GS domain) (77). Specificity of signal propagation to Smad molecules, the main downstream components in serine/threonine kinase receptor signaling, is determined by type I receptors (reviewed in refs 29, 101). More specifically, a region flanking β strands 4 and 5 in the kinase domain, designated L45 loop, confers a high degree of specificity in Smad interaction with the type I receptors (78, 80). The L45 loop is highly conserved among type I receptors with comparable signaling specificity, but differs significantly between BMP and TGF-β or activin type I receptors (79, 80). Swapping the L45 loop between TβR-I and BMPR-IB, followed by ligand-stimulation, was shown to result in exchange of Smad1 vs. Smad2 recognition specificity, accompanied by a switch in transcriptional responses (79, 80). However, differential effects between the two BMP type I receptors have been described (102–104). CDMP-1 and CDMP-2, which both interact with BMPR-IB and BMPR-II, differentially regulate osteogenesis (105, 106). Thus, in addition to a role for the L45 loop, other mechanisms must contribute to specify downstream signaling after activation of type I receptors.

It has been shown that the cytoplasmic juxtamembrane region of TβR-I is involved in specifying signal transduction, since mutations of certain serine or threonine residues within this region selectively impair TGF-β-mediated growth inhibition, but do not affect TGF-β-induced PAI-1 or fibronectin synthesis

(107). However, *in vivo* phosphorylation of these sites by TβR-II has not yet been reported. Mutation of Ser165, located amino-terminal of the GS domain, results in potentiati n of TGF-β-mediated growth inhibition and extracellular matrix induction but diminishes TGF-β-induced apoptosis (108). Thus, additional residues in TβR-I appear to modulate distinct pathways triggered by TGF-β.

In certain cell systems it has been observed that the expression level of type I receptors correlates with extracellular matrix production and gene transcription, whereas the expression level of type II receptors correlates to the growth inhibitory response, suggesting participation of type I and type II receptors in different signaling pathways. However, the observations made are all compatible with an alternative interpretation, which is that all signals are initiated by activation of the type I receptor and that different activation thresholds of the signaling pathways leading to growth inhibition vs. gene transcription underlie the apparent differential modes of signaling by type I and type II receptors (109).

INTRACELLULAR SIGNALING BY TGF-β SUPERFAMILY MEMBERS

Receptor-activated Smads and common-partner Smads as mediators of TGF-β signal transduction

Genetic studies in *Drosophila* and *Caenorhabditis elegans* have led to identification of a conserved family of intracellular signal transducers for TGF-β superfamily members. The founding member, *Mothers against dpp* (*Mad*) (110, 111), was isolated from a genetic screen for enhancers of a weak Dpp maternal phenotype. Subsequently, three *Mad* homologues, called *Sma-2, Sma-3, and Sma-4*, were picked up in *C. elegans* by searching for genes whose mutations caused the same small body-size phenotype as observed for mutant *Daf4*, a type II serine/threonine kinase receptor (112). At present, eight different Sma- and Mad-related proteins have been identified in mammals, and are termed Smads (**Fig. 3A**). They can be subdivided into three distinct subclasses: receptor-activated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (anti-Smads). R-Smads and Co-Smads are homologous in their amino- and carboxyl-terminals, called the MH1 and MH2 domains, respectively. These domains are connected by a proline-rich linker region. R-Smads contain SSXS phosphorylation motifs in their own carboxy termini.

Smad1, Smad5, and Smad8 are involved in BMP signaling and become phosphorylated by ActR-I, BMPR-IA, or BMPR-IB (113–117). Smad2 and Smad3 are mediators of TGF-β and activin signaling

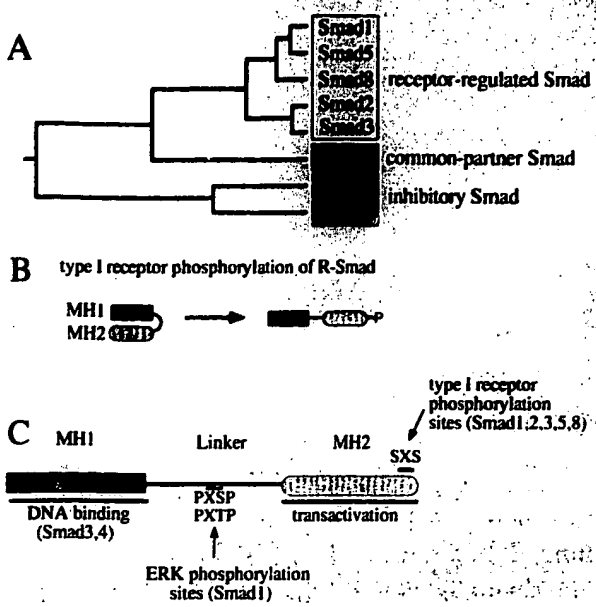

Figure 3. The Smad family of intracellular signal transducers for TGF-β superfamily proteins. *A)* A phylogenetic analysis of mammalian Smad proteins. *B)* The Mad homology (MH) 1 and MH2 domains have affinity for each other; type I receptor-mediated phosphorylation of R-Smads at their carboxy termini may induce a conformation change, thereby relieving the autoinhibitory effect of MH1 on MH2 and vice versa. *C)* Schematic structure of R-Smad and Co-Smad and their functional domains. Direct DNA binding is mediated through the MH1 domain whereas MH2 has transcriptional activation activity. The sites of phosphorylation in R-Smads by type I receptors at the extreme carboxy terminus and by ERK in linker region are indicated.

and interact with and become phosphorylated by TβR-I or ActR-IB (118–121). Consistent with these findings, injection of BMP-Smads into *Xenopus* animal caps results in ventralization of mesoderm (115, 116, 122, 123), and ectopic expression of Smad2 in *Xenopus* embryos induces dorsal mesoderm and secondary axis formation analogous to activin/Vg-1-like responses (122, 123). Smad2 null mice lack anterior-posterior specification and fail to develop mesoderm (124, 125). The phenotype of Smad2 knockouts is strikingly similar to Nodal knockouts and suggests that they cooperate in regulation of gastrulation (124). Mutant mice lacking Smad3 expression have been reported to be viable and fertile; however, Smad3 −/− animals were found to exhibit limb malformations (126), have a defect in immune function (127), and develop colon carcinomas 4–6 months after birth (128). Smad3 null cell lines showed strongly impaired TGF-β responsiveness and indicated an important role for Smad3 in TGF-β-mediated inhibition of cellular proliferation (126). The Smad3 knockout phenotype observed by Zhu and co-workers (128) classifies Smad3 as a tumor suppressor gene, although Smad3 mutations have not yet been detected in tumorigenesis (129).

TGF-β-mediated phosphorylation of Smad1 has

been observed in breast tumor cells, but the receptor by which this phosphorylation is mediated has not been determined (130). Smad5 phosphorylation in response to TGF-β was detected in CD34+ cells (131). ALK-1 has been shown t bind TGF-β and to phosphorylate and activate BMP-receptor-regulated Smads (113, 114; T. Imamura, P. ten Dijke, and K. Miyazono, personal communication), and thus could possibly mediate TGF-β-induced Smad phosphorylation in these cells.

Three Co-Smads have been characterized: mammalian Smad4/DPC4 (deleted in colon carcinoma; ref 132), *C. elegans* Sma-4 (112), and *Drosophila* Medea (133). Co-Smads play a critical role in signaling by TGF-β superfamily members, illustrated by the absence of TGF-β signaling and R-Smad functioning in Smad4-deficient tumor cell lines, as well as by the synergistic action of Smad4 with R-Smads in transcriptional activation of target genes and induction of mesoderm (121, 134–137). The importance of Smad4 functioning is also shown by its tumor suppressor activity; mutation or deletion of Smad4 is often associated with tumorigenesis (132, 138–141). Cell lines expressing Smad4-mutants found in human cancers failed to transcriptionally activate transfected luciferase reporters containing Smad binding elements (140). Mice lacking expression of Smad4 show a BMP knockout phenotype, characterized by growth retardation and gastrulation defects (142). However, the abnormalities in mesoderm development of Smad4 null mice could be overcome by the presence of wild-type extraembryonic tissue, indicating that Smad4 is not critical for early gastrulation (142). Although several explanations are possible, one reasonable argument is that a yet unidentified Co-Smad is involved in mediating early embryonic differentiation induced by members of the TGF-β superfamily. At later stages of development, Smad4 is important for anterior specifications (142).

Additional indications for the possible existence of novel Co-Smads comes from studies in *Drosophila* where Medea, in contrast to Mad, is dispensable for oogenesis (143). An explanation for the fact that Mad knockouts have a more severe phenotype than Medea knockouts might be that Mad is able to signal certain responses independent of Medea. Furthermore, mutations in Medea efficiently block signaling by Saxophone (Sax) but fail to fully suppress signaling via Thick Veins (Tkv), both of which are Dpp type I receptors (133). Although Medea might be abundantly present to allow Tkv signaling even when hypomorphic alleles are expressed, the inability to fully abrogate Tkv signaling might also reside in the fact that another, yet unidentified, Co-Smad is involved in Tkv signaling or that Tkv can signal independent of Medea. Besides the XSmad4 ortholog, another Smad4/Medea-like protein was recently identified in *Xenopus* (144, 145; C. Hill, personal communication)

Mechanisms controlling Smad activati n

R-Smads occur as monomers in the cytoplasm (146) and, after ligand-induced type I receptor activation, specifically interact with the L45 loop in the kinase domain of type I receptors (79, 80). Recruitment of R-Smads to the TGF-β receptors is promoted by the action of SARA, a FYVE zinc finger domain-containing protein that interacts with both TGF-β receptors and Smad2 or Smad3 (Fig. 2) (147, 148). The highly conserved L3 loop of R-Smads, which protrudes from the MH2 core domain as observed in the Smad4-MH2 crystal structure (139), is involved in specifying type I interactions with TβR-I and BMPR-I (150). This loop varies between Smad1 and Smad2 in only two amino acids, and exchanging these residues between Smad1 and Smad2 enables their binding to TβR-I and BMPR-I, respectively (79, 149). The α-helix 1 of Smad1, located near the L3 loop in the 3 dimensional structure, has been shown to be important for the interaction of Smad1 with ALK-1 (114).

After type I receptor interaction, R-Smads become activated by phosphorylation of their carboxyl-terminal SS(M/V)S motifs (Fig. 3*B*) (119, 150–152), which triggers their homodimerization and heteromerization with Co-Smads (134, 153) or other R-Smads (121, 146). This leads to the speculation that homomeric R-Smads, heteromeric R-Smads, and R-Smad/Co-Smad complexes might control different biological responses.

In contrast to R-Smads, Co-Smads do not contain the carboxyl-terminal phosphorylation motif SSXS and are not phosphorylated by the activated type I receptor (119, 120). Although the L3 loop of Co-Smads shares high amino acid similarity with the L3 loop of R-Smads, Co-Smads do not interact with type I receptors (120); instead, their L3 loops are important for heteromerization with phosphorylated R-Smads (139). Resolution of the crystal structure of the Smad4-MH2 domain and analyses of Smad-deletion mutants (139) have indicated that the MH2 domains of R-Smads and Co-Smads are involved in formation of homo-and heteromeric trimers. After association of R-Smads with Co-Smads, the heteromeric complexes are translocated into the nucleus (see Fig. 5).

Domains controlling nuclear translocation

Whereas ligand-induced nuclear translocation of R-Smads is not dependent on expression of Co-Smads (136), Co-Smads require association with activated R-Smads in order to enter the nucleus (136, 151). A carboxyl-terminal truncation of Smad4, correspond-

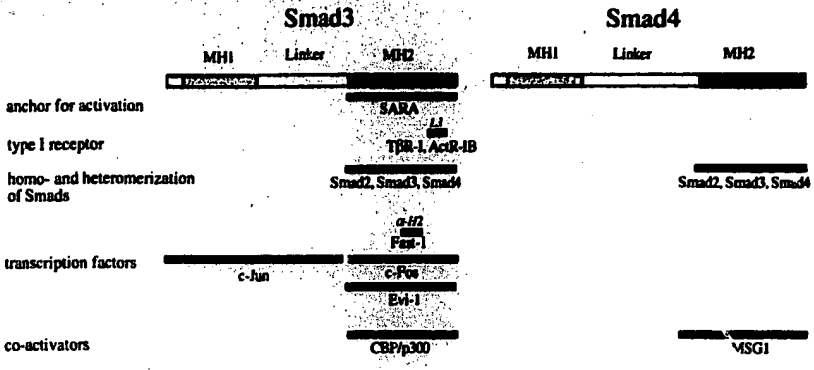

Figure 4. Smad interacting proteins. Overview of the interacting proteins for Smad3 and Smad4. All proteins that interact with Smad3 also interact with Smad2 except for c-Jun, c-Fos, and Evi-1. The interaction of TβR-I with Smad2 or Smad3 occurs through the L3 loop in the MH2 domain, and association of FAST-1 with Smad2 or Smad3 is determined by an exposed α-helix 2 in MH2 domain.

ing to a mutation of Smad4 found in pancreatic tumors (132) and identified in an inactive form of Mad in *Drosophila* (111), dominant-negatively interferes with nuclear transport of all R- and Co-Smads (137). In contrast, a comparable mutation in R-Smads does not affect their nuclear localization (137). This indicates that the carboxyl-terminal part of Co-Smads is required for nuclear trafficking (137), whereas phosphorylation of R-Smads, though required for their functionality, is not obligatory for nuclear transport. How these mutant Co-Smads interfere with the nuclear transport function of R-Smads needs to be further studied. Removal of the NH_2 terminus and linker region of R-Smads results in strong, ligand-independent nuclear localization (122, 137), whereas an additional carboxyl-terminal truncation of the remaining MH2 domain results in cytoplasmic accumulation at the nuclear membrane (137). Presumably, ligand-induced heteromerization results in a conformational change of the Smad molecules, thereby exposing nuclear translocation signals in R- and Co-Smads. Whether the cytoplasmic residence of R-Smads and Co-Smads in the absence of ligand is due merely to autoinhibition of the MH2 effector domain by association with the MH1 domain (154), or whether a cytoplasmic tether exists that anchors R- and Co-Smads in the cytoplasm in the absence of ligand, is not clear (137).

R-Smads and Co-Smads as sequence-specific transcriptional activators

Once in the nucleus, R-Smads and Co-Smads are involved in transcriptional regulation of target genes (Fig. 3*C*) (reviewed in ref 155), and the MH1 and MH2 domains differentially contribute to this property. Direct binding of Smads to DNA was first shown by the interaction of the *Drosophila* Mad-MH1 domain with specific sequences in the promoter of the Dpp target gene *vestigial* (156). A prerequisite for the interaction of Mad, Smad3, but not Smad4, with DNA is the dissociation of the MH2 domain from the MH1 domain, which is achieved by ligand-induced phosphorylation of the SSXS motif in R-Smads (154, 156). Thus, the MH2 domain can exert an autoinhibitory action on the MH1 DNA binding domain.

In several promoters of genes induced by TGF-β signaling like *type VII Collagen*, *JunB*, and *PAI-1* (157, 158, 160), unique elements have been identified with which Smad3 and Smad4 physically interact. The sequences of the Smad binding elements (SBEs) in these promoters are highly related and indicate an AGAC-containing motif as binding site for Smad3 and Smad4. This sequence was also picked up in a random screening for Smad interacting DNA sequences (161), and mutation of these SBEs in the PAI-1 promoter impairs TGF-β responsiveness (160).

Crystalization of the Smad3 MH1 domain bound to an optimal Smad binding sequence revealed that the so-called β-hairpin loop, protruding from the MH1 core domain, interacts with DNA (162). The β-hairpin loop is highly conserved among R-Smads and Co-Smads. However, Smad2 contains an extra exon preceding this region, encoding 30 amino acids, that might interfere with the correct conformation of the β-hairpin loop, in contrast to Smad3 and Smad4. Smad2 has not yet been shown to interact with DNA. Removal of exon 3 in Smad2 enables its efficient binding to DNA (163, 164).

In several TGF-β target genes, multiple copies of SBEs can be identified, often located in close proximity to sites for other transcription factors (158, 160, 165–167). Smads have been shown to mediate transcriptional activation by interaction with other regulatory promoter units, like the TPA-responsive elements (TREs)/AP-1 sites in the collagenase promoter, which actually overlap in sequence with SBEs (158, 166). Smads cooperate with the transcription factor TFE3 in the regulation of the *PAI-1* gene (168). The p21/WAF1/Cip1 and p15/INK4B gene promoters lack SBEs, but TGF-β transcriptionally regulates these genes via the Sp1 site (169–171). It has been suggested that the interaction of Smads with additional transcription factors such as AP-1 (**Fig. 4**; 166, 172, 173), forkhead activin signal transducers (FAST; 165, 167, 174), or Sp1 (169, 171) confers additional DNA binding specificity to the Smad-containing transcriptional complex (see below).

R-Smads and Co-Smads as transcriptional (co-)activators

Studies with *Xenopus* explants, in which injection of the Smad2-MH2 domain fully induced dorsalization of mesoderm and secondary axis formation analogous to activin signaling (122), suggested an effector function for MH2 domains. Furthermore, transcriptional activation properties of R- and Co-Smads were shown by fusion constructs of Smad1-MH2 or Smad4-MH2 domains with Gal4-DNA binding domains (175). Heteromerization of Co-Smads with R-Smads is obligatory for their transcriptional activation properties (136, 153, 176).

Several domains in Smad4 have now been identified to contribute to its important role in signaling by members of the TGF-β superfamily. As mentioned above, the MH1 domain enables the interaction of R-Smad/Co-Smad complexes with DNA, which is of particular importance for Smad2-Smad4 heteromeric complexes (136) since Smad2 is not endowed with DNA binding capacities. The MH1 domain autosuppresses the MH2 effector domain (154), and heteromerization of Co-Smads with phosphorylated R-Smads has been suggested to release the inhibitory association (177). Studies in which chimeras between Smad1 and Smad4 were tested revealed that the proline-rich linker region of Smad4 contains a domain that is important for its transcriptional activity mediated by the MH2 domain (178). This Smad activation domain (SAD) cooperates functionally with the transcription coactivator MSG1 (178, 179). MSG1 strongly enhances Smad4 transcriptional activity, provided that Smad4 is present in a heteromeric complex with R-Smads (179). Smad heteromerization possibly introduces a configuration that allows *trans*-activators to associate and endow Smad4 with transcriptional potencies (179).

Smads interact with transcription factors

The first evidence that Smads interact and cooperate with transcription factors comes from the interaction observed between Smad2, Smad4, and *Xenopus* FAST-1 (XFAST-1), making a complex that interacts with the activin responsive element (ARE) in the *Xenopus* Mix2 promoter (see Figs. 4, 5) (136, 180, 181). Human FAST-1 and mouse FAST-2 (mFAST-2) have been identified as well, but share little sequence homology with XFAST-1 (165, 167, 174). FAST-1 efficiently interacts with DNA, and Smad2 and Smad4 contribute in providing additional DNA interactions as well as essential transcriptional activation properties (136, 167, 174, 180, 181). Phosphorylation of the Smad2 SSMS motif is required for interaction of α-helix 2, exposed from the MH2-trimer structure, with FAST-1 (136, 167, 180, 181).

Whereas Smad2/Smad4/mFAST-2 complexes drive transcriptional activation of the goosecoid reporter, Smad3/Smad4/mFAST-2 complexes were found to suppress promoter activation, presumably because Smad3 and Smad4 share the same DNA binding site (165). This suggests that the relative expression levels of Smad2 and Smad3 in a cell might determine the outcome of certain biological responses induced by TGF-β.

Additional evidence for differential roles of Smad2 and Smad3 comes from observations in human keratinocytes, where TGF-β stimulates Smad2 and Smad3 to comparable extents while activin predominantly activates Smad3 (182). In human lung epithelial cells, Smad3 more potently induces apoptosis compared to Smad2 (183).

Cooperation of Smads with the AP-1 complex

Characterization of Smad binding sites in a collagenase promoter revealed that Smads can mediate transcription via 12-O-tetradecanoyl-13-acetate (TPA) responsive elements (TREs; 120, 121, 158), to which the transcription factor AP-1, a dimer of c-Jun and c-Fos, binds. These AP-1 binding sites overlap in sequence with consensus Smad binding sites (158). Smad3 directly interacts with TRE, and Smad3 and Smad4 activate the TGF-β-inducible transcription of TRE-Luc reporter in the absence of c-Jun and c-Fos (166). However, TGF-β-induced reporter activity was further enhanced in the presence of c-Jun-c-Fos heterodimers (166). After TGF-β-induced Smad activation, Smad3 and Smad4 can interact with JunB, c-Jun, and JunD as well as with c-Fos (166, 172). By footprint analysis and electromobility shift assays, it was shown that c-Jun and Smad3 can bind simultaneously to overlapping sequences in the TRE (166). Crystallographic analysis of the Smad3 MH1 domain bound to DNA indicated that a conformational change of the NH_2 terminus of c-Fos would allow the multimeric Smad-AP-1 complex to interact with a single AP-1 site (162). This cross talk might be an important level of coordination between Smads and MAPK/JNK signaling.

Interaction with the coactivators p300/CBP

Insight into how Smads mediate their transcriptional activating function was recently unraveled by the interaction of R-Smads with the coactivators p300 and CREB binding protein (Fig. 4) (CBP; 176, 184–187). These coactivators contribute to transcriptional activation by loosening the chromatin structure or by increasing the affinity of certain transcription factors for DNA through their intrinsic (or associated) acetyltransferase activity. In addition, they act as a bridging factor between transcription factors and the basal transcription machinery (184

and references therein). In view of the importance of Smad4 for transcriptional activation of the Smad-p300/CBP complex, Smad4 might function as a coactivator as well (176). The interaction between the MH2 domain of R-Smads and CBP/p300 is triggered by ligand-induced phosphorylation of the carboxyl-terminal SSXS motif (176, 184, 186). Phosphorylation most likely induces a conformational change in the R-Smad molecule, which then results in exposure of the CBP/p300 binding site; deletion of the NH_2 terminus and part of the carboxy terminus, leaving the CBP binding site intact, enhances the affinity of Smad3 for CBP/p300 (184). The carboxyl-terminal region of CBP is required for Smad interaction (176, 184, 186) and can associate with other transcription factors like the adenoviral oncoprotein E1A. E1A is known to interfere with TGF-β-mediated signaling (188, 189) and is shown to abrogate Smad-mediated transcriptional activation (176, 184, 186), most likely by direct competition with Smads for binding to CBP/p300 (188, 189).

Negative regulation of Smad function

Activin and BMPs have been shown to compete which each other in *Xenopus* mesoderm development due to limiting amounts of Smad4 (190). In view of the limited amounts of CBP/p300 in the cell (184) and the observed competition between different signal transduction pathways at the level of coactivator availability (191), competition between members of the TGF-β superfamily as observed in embryogenesis can also be expected to occur at the level of CBP/p300.

Interaction of the first zinc finger domain of the nuclear protein Evi-1 with the MH2 domain of Smad3 interferes with binding of Smad3 to DNA (Fig. 4), thereby repressing transcriptional activity of Smad3 as well as growth inhibition (192). Evi-1 shows a spatial and temporal expression pattern during mouse embryogenesis, suggesting it could play a regulatory role during development (193).

Inhibitory Smads

Mammalian Smad6, Smad7, and *Drosophila* Dad have been characterized as inhibitors of TGF-β signal transduction (**Fig. 5**) (194–198). Whereas their amino-terminal domains are highly diverse and share only weak similarity to other Smads, they are homologous to the R- and Co-Smads in their MH2 domains. However, inhibitory Smads lack the carboxyl-terminal SSXS phosphorylation motif, which may enable them to stably associate with type I receptors and to interfere with receptor binding and activation of R-Smads. Phosphorylation of the type I receptors by the type II receptors is essential for inhibitory Smad

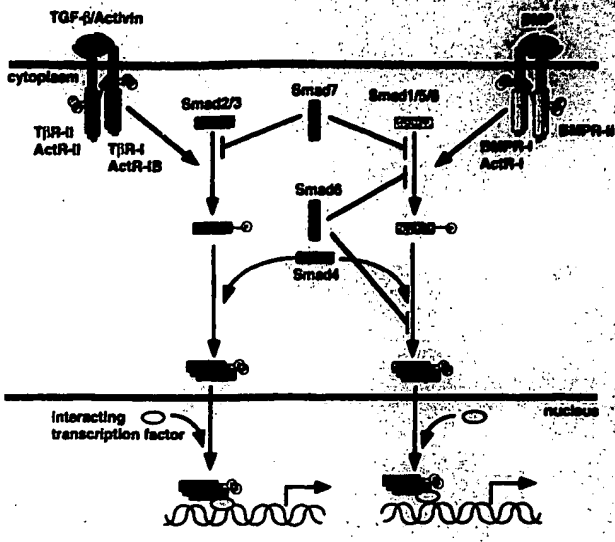

Figure 5. TGF-β superfamily signaling through signal-transducing Smad and inhibitory Smad proteins. After type I receptor activation, R-Smads become phosphorylated, form homomeric complexes with each other, and assemble into heteromeric complexes with Co-Smad, Smad4. Trimers are shown, but hexamers have not been excluded. The stoichiometry between the components is unclear. The heteromeric complexes translocate into the nucleus, where they regulate, in combination with other transcription factors, transcription of target genes. Smad2, Smad4, and FAST-1 are components of an activin-responsive factor that interacts directly in an activin-dependent manner with an activin response element of the *Mix.2* promoter. Inhibitory Smads act opposite from R-Smads by competing with them for interaction with activated type I receptors or by directly competing with R-Smads for heteromeric complex formation with Co-Smad. Smad7 appears to be a general inhibitor of TGF-β superfamily signaling whereas Smad6 preferentially inhibits BMP-induced responses.

association (194, 196, 199) and the MH2 domain of anti-Smads suffices in exhibiting the inhibitory effect (194, 196, 199, 200), as manifested by inhibition of R-Smad phosphorylation and abolition of their heteromerization with Co-Smad and nuclear translocation, as well as abrogation of growth factor-induced transcriptional responses and growth inhibition (194–196, 198–202). It has been reported that Smad6 can exert its inhibitory effect via an alternative mode that involves BMP-induced association of Smad6 with phosphorylated Smad1, thereby competing with activated Smad1 for heteromerization with Smad4 (200) (Fig. 5).

The expression of the inhibitory Smads is quickly induced upon stimulation by TGF-β superfamily members and thereby provides an autoinhibitory mechanism in TGF-β signaling (196, 197, 201, 203). The human Smad7 promoter contains an SBE consensus site that, when fused to a luciferase gene, induces luciferase activity after TGF-β stimulation (G. Brodin and R. Heuchel, personal communication). Thus, after receptor activation, R- and Co-

Smads might be directly involved in transcriptional regulation of the Smad7 gene.

The MH1 domain of inhibitory Smads might play a role in specification of their inhibitory actions: *Xenopus* and mouse Smad7, which share 96% identity in their carboxyl-terminal domain but only 51% in the amino-terminal region, exert differential effects in inhibition of TGF-β and activin signaling (199). Mouse Smad7 inhibits TβR-I-induced phosphorylation of Smad2 and Smad3 (194, 196, 199), BMPR-IA and BMPR-IB-mediated phosphorylation of Smad1 and Smad5 (199), and ActR-I mediated phosphorylation of Smad1 (199); it therefore appears to be a common inhibitor of R-Smad activation. Whereas Smad6 has been implicated in inhibition of TGF-β signaling (194, 204), it appears to play a more pronounced role in inhibition of BMP signaling (Fig. 5) (195, 200, 201, 203, 205, 206). The mechanism of action for inhibitory Smads in TGF-β superfamily signaling is evolutionarily well conserved; it has recently been shown that Dad, which is a Dpp-inducible inhibitory Smad in *Drosophila* (197), interacts with Tkv and thereby prevents binding and phosphorylation of Mad by Tkv (198).

The *in vivo* relevance of Smad6 and Smad7 is shown in several systems. Smad6 and Smad7 were initially identified as genes induced by shear stress in vascular endothelial cells (204). Smad7 inhibits activin-induced dorsal mesoderm formation and interferes with inhibition of activin-mediated growth arrest and apoptosis in B-cells (206). Smad6 and Smad7 antagonize BMP-induced mesoderm development, have neural-inducing potencies in *Xenopus*, and inhibit BMP-induced growth arrest and apoptosis in B cells (200, 205–207).

Alternative functions for Smad6 and Smad7 may exist as well. In *Xenopus*, XSmad6 was found to be partially or completely restricted to the nuclei in most cells (207). In mammalian cells, Smad7 has been located in the nucleus in the absence of ligand, but rapidly accumulated in the cytoplasm after TGF-β stimulation (202). In accordance with its importance for inhibition of R-Smad signaling, the inhibitory Smad carboxyl-terminal tail also harbors domains required for transport across the nuclear membrane (202). A nuclear localization of inhibitory Smads might be required to allow phosphorylation of R-Smads after receptor activation; the mechanism whereby Smad7 is translocated to the plasma membrane after TGF-β receptor activation is unknown. An interesting possibility, which remains to be elucidated, is that inhibitory Smads in addition function in transcriptional regulation. Differential compartmentalization of the inhibitory Smads, in combination with induction of their expression after signaling by TGF-β superfamily members, and the fact that inhibitory Smads can selectively eliminate particular R-Smad pathways provide a tightly controlled cell-autonomous regulatory mechanism.

Modulation of Smad signaling by MAP kinase pathways

The mitogen-activated protein kinase kinase kinase TAK1 (TGF-β activated kinase 1) has been shown to be phosphorylated and activated upon TGF-β or BMP-4 stimulation (208), and can mediate transcriptional activation of a luciferase reporter driven by a TGF-β-inducible element of the PAI-1 promoter containing three AP-1 sites. This effect is strongly enhanced by the TAK1-activator TAB1 (209). The *in vivo* relevance of the cooperative functioning of TAK1 and TAB1 was demonstrated in *Xenopus* mesoderm development in which TAK1 and TAB1 promoted ventral mesoderm induction and perturbed neural differentiation, thereby substituting BMP signaling (210). The human X-chromosome-linked inhibitor of apoptosis protein (XIAP) can interact with both BMP receptors as well as with TAB1 and enhances ventralization of *Xenopus* embryos in a TAB1-TAK1-dependent manner (211).

Using the transmembrane and cytoplasmic domain of BMPR-IA as bait in yeast two-hybrid screens, BRAM1 (BMP receptor-associated molecule) was identified. The carboxyl-terminal region of BRAM1 is responsible for specific interaction with BMPR-IA and does not associate with the kinase domain of TβR-I (212). Furthermore, this region in BRAM1 is also responsible for interaction with TAB1 (212). Expression of GST-coupled BMPR-IA, HA-tagged BRAM1, and myc-tagged TAB1 in COS7 cells showed that the three proteins form a ternary complex. BRAM1 might function as an adaptor protein for positioning TAB1 in close proximity of BMPR-IA kinase domain (212). The role of BRAM1 in activation of the TAB1/TAK1 signaling cascade, as well as its possible effect on Smad-receptor interaction and Smad-activation is not clear at present.

A downstream phosphorylation target in the MAPK cascade triggered by TAK1 is mitogen-activated protein kinase kinase 4 (MKK4)/stress-activated protein kinase/extracellular signal-regulated kinase SEK1 (208, 213), which is involved in the stress-activated protein kinase (SAPK)/c-Jun NH_2-terminal kinase (JNK) pathway, finally leading to activation of c-Jun. In that JNK activity induced by hematopoietic progenitor kinase-1 (HPK-1) can be perturbed by expression of a kinase inactive form of TAK1, there are indications that TGF-β might exert JNK activation via the HPK1-TAK1-SAPK/JNK route (214). The small Rho-like GTPases Rho, Rac, and cdc42, which signal via the SAPK/JNK pathway, have also been shown to be important for certain aspects of TGF-β signaling (215, 216).

The SAPK/JNK pathway has been shown to affect TGF-β signaling via the Smad-pathway, since overexpression of dominant negative members of the SAPK/JNK pathway prevent both TGF-β- and Smad4-mediated transcriptional response (217). The interface of SAPK/JNK- and Smad-signaling might be at the level of interaction between c-Jun and Smad3, since these transcription factors have been shown to associate after TGF-β stimulation and synergize in activating a transcriptional reporter containing AP-1 binding sites (163, 166).

Other levels of interaction between MAPK pathways and Smad signaling have been elucidated. In the linker regions of R-Smads that connect the MH1 DNA binding domain with the MH2 transcriptional domain of receptor-activated Smads, several ERK recognition sites (PXSP, or PXTP motifs) (Fig. 3*B*) and JNK motifs (XXSP) are present in the linker region of R-Smads. Phosphorylation of these sites in Smad1 by EGF or HGF has been shown to circumvent nuclear translocation of Smad1 and indicates a role for ERK signaling in the modulation of TGF-β signaling (218). On the other hand, EGF and HGF have been shown to mediate signaling via Smad2, activated by kinases downstream of MEK1 (219). Furthermore, deletion of the carboxyl-terminal phosphorylation motif SS(V/M)S abrogates HGF-induced Smad1 or Smad2 phosphorylation, their nuclear translocation, and HGF-induced activation of a Smad responsive reporter construct, indicating that these residues are important for Smad functioning in both TGF-β and HGF or EGF signaling (219). It is not known under which conditions the ERK signaling inhibits TGF-β family/Smad-induced responses or mediates Smad-dependent pathways.

Analogously, differences in cellular context might underlie the apparently contradicting reports on the role of Ras/MAPK in TGF-β signaling in different tumor cell systems. Phosphorylation of ERK sites in the linker region of Smad3 by activated Ras, MEK1, or v-HA-Ras-transformed mouse mammary epithelial EpH4 cells inhibits TGF-β/Smad-mediated transcriptional responses, Smad nuclear translocation, and growth inhibition (220). In contrast, TGF-β rapidly induces Ras/MEK signaling in intestinal epithelial IEC1 4 cells; this signal transduction pathway cooperates in TGF-β mediated Smad-signaling, presumably by phosphorylation of Smad1 on the four ERK-sites in the linker region (221).

CONCLUSIONS AND PERSPECTIVES

Recent genetic and biochemical studies in *C. elegans*, *Drosophila*, *Xenopus*, and mammals have now firmly established the TGF-β/Smad pathway as a pivotal means for intracellular signaling of TGF-β superfamily members (reviewed in refs 29, 101). On ligand-induced heteromeric complex formation of distinct type I and type II receptors, particular R-Smads transiently interact with and become phosphorylated by the activated type I receptor kinase. Subsequently, R-Smads assemble with Co-Smads into heteromeric complexes that translocate into the nucleus, where (in combination with other transcription factors) they regulate the transcription of target genes.

One TGF-β superfamily member can couple with multiple type I receptors and multiple Smads, each mediating a distinct set of responses (reviewed in refs 29, 101). Thus, the receptor and Smad expression profile in the target cell is an important factor that decides which particular cellular responses are induced by a TGF-β superfamily member. In addition, the repertoire of transcription factors that are present in the cell with which the Smads can interact is a critical determinant. Although multimerized Smad binding element is sufficient to drive TGF-β-induced transcription (reviewed in ref 155), emerging promoter analyses of TGF-β superfamily target genes indicate that transcriptional regulation is achieved by cooperation of Smads with other transcription factors. Complex formation of Smads with these transcription factors may occur independent of DNA binding or require DNA binding for additional selectivity (reviewed in ref 155).

The TGF-β/Smad pathway has been implicated in many responses, including growth inhibition, differentiation, and many transcriptional responses. An important issue that needs to be addressed is the requirement of particular Smads in these responses. In addition, it will be important to explore the possibility that Smad-independent signaling also occurs. TGF-β-induced transcription of the fibronectin gene can occur independent of Smad4, but requires the JNK pathway and activation of cAMP-responsive element by cJun/ATF2 heterodimer (222).

An important task for the future is to determine the genetic programs that are triggered on challenging different cell types with different TGF-β family members. This will become feasible by applying the newly developed functional genomics and proteomics technology and thereby determine the time- and dose-dependent effects of TGF-β family members on the expression of thousands of genes/proteins simultaneously. In addition, changes in the expression patterns on ectopic expression of constitutively active or dominant negative versions of signaling components or on ligand stimulation in cells deficient in such components can be measured. The target genes/proteins identified will need experimental follow-up to evaluate their importance in various biological responses. Moreover, the involvement of different type I receptors and Smad molecules in

various responses induced by TGF-β family member can be elucidated using this methodology.

The multifunctional character of TGF-β superfamily members implicates a need for tight control of their activities. Indeed, both positive and negative (feedback) regulatory mechanisms have been observed at nearly every step in the TGF-β superfamily signaling cascade, from the release of biologically active ligand to the Smad-mediated transcriptional effects. Cross talk of the TGF-β/Smad pathway with other pathways will be a theme of many future studies, e.g., how other signaling inputs affect the activity, expression, stability, or subcellular localization of TGF-β superfamily receptors and Smads. In addition, the pleiotropic action of TGF-β superfamily members has driven the evolution of multiple closely related TGF-β superfamily members with different expression and activation patterns. The mammalian TGF-β superfamily will likely continue to grow until the complete human sequence has been elucidated at the beginning of the next century.

Much of our insight into signaling mechanisms has come from the genetically accessible model organisms *Drosophila* and *C. elegans* (reviewed in refs 29, 101). In *Drosophila*, dpp signals through two type I receptors—saxophone and thick veins—and their downstream effector, Mad. Recently, an activin/TGF-β signal transduction pathway was identified in *Drosophila* (223, 224). Activation of the activin type I receptor Atr-I stimulates dSmad2-dependent pathways. The type II receptor punt and Medea are shared components between the Dpp and activin/TGF-β pathway. *C. elegans* research obtained a boost by the recent completion of its entire genome sequence (reviewed in ref 225). This vertebrate has four TGF-β-like ligands (including Daf-7 and Daf-1), one type II receptor (Daf-4), and two type I receptors (Daf-1 and Sma-6). Thus, Daf-4 is required for both pathways and type I receptors to determine signaling specificity (226); Daf-1 is important in dauer larva formation and Sma-6 for body size determination and male tail development. Six Smad proteins are present in the *C. elegans* genome: Sma-2, Sma-3, and Sma-4 have been implicated in the Daf-7/Daf-1 pathway, and the Smad mediators Daf-8, Daf-14, and Daf-3 in Dbl-1/Sma-6-induced signaling responses. *Drosophila* and *C. elegans* will continue to be extremely important in elucidating the molecular mechanisms that underlie TGF-β superfamily signaling.

In mammals, the physiological significance of interactions between ligands and receptors, between receptors and Smads, and between Smads and Smad-interacting proteins, as well as the importance of the signaling pathways in which they act, need to be validated through comparison of the phenotypes of mice deficient in a particular TGF-β superfamily member, receptor, Smad, or target gene. In those cases where a null mutation leads to an early embryonal lethal phenotype, conditional knock-out approaches will be required to study the role of this component in late development or adult tissues. In addition, transgenic approaches in mice with dominant negative or constitutively active forms of TGF-β superfamily signal transducers under inducible or tissue-specific promoters will provide important information regarding issues of signaling specificity and diversity in different cellular contexts.

The improved understanding by which TGF-β superfamily members elicit and regulate different responses will be essential in the design of new therapeutic approaches for various diseases caused by deregulated activity of TGF-β family members. For example, antagonists of TGF-β could be applied in various types of fibrosis that are due to TGF-β overactivity and agonists of TGF-β in diseases in which enhanced activity is beneficial, such as wound healing or immunosuppression. In most cases it will be advantageous not to inhibit or activate all activities of TGF-β superfamily members. By understanding the molecular mechanisms that underlie specificity, diversity, and regulation in TGF-β superfamily signaling, it will be possible to screen for pharmacological compounds that inhibit or mimic only defined activities of TGF-β superfamily members. FJ

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ORIGINAL ARTICLE

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Sequential protocol biopsies from renal transplant recipients show an increasing expression of active TGF β

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Abstract Chronic allograft nephropathy (CAN) is a major cause of graft loss after renal transplantation. Implicated in the pathogenesis of this complication is overproduction of the cytokine transforming growth factor beta (TGF β). In this study we measured changes in CAN's expression in stable patients early after transplantation, and studied links with established risk factors for CAN, such as delayed graft function, acute rejection, and cyclosporine exposure. We took biopsies from 40 renal allografts at time of transplantation (pre-perfusion), and then, using ultrasound guidance, at 1 week and 6 months after transplantation. An immunofluorescence technique was used to stain sections for active TGF β. These were then assessed by semi-quantitative scanning laser confocal microscopy. There was very little variation in active TGF-β expression among patients in their pre-perfusion biopsies. Expression had increased by 1 week and then very significantly by 6 months ($P < 0.0001$). Patients who suffered delayed graft function had increased TGF-β expression at both time points. There was no difference regarding donor type, acute rejection, and immunosuppressive drug (cyclosporine or tacrolimus). There was no correlation between the amount of TGF-β expression at any time-point and isotope glomerular filtration rate (GFR) at 12 months. This study demonstrated that in a group of stable renal allograft recipients, TGF-β expression in the kidney increased after transplantation. As the study used protocol biopsies, this increase is unlikely to be due to acute events, and probably represents a genuine increase.

Keywords Chronic allograft nephropathy · Transforming growth factor beta · Protocol biopsies

Introduction

As the use of modern immunosuppressives has resulted in less early graft loss due to acute rejection, many renal transplants succumb to a gradual deterioration several years later. This is characterised histologically by fibrosis affecting all compartments of the kidney, and is most commonly described as chronic allograft nephropathy (CAN). While the aetiology is incompletely understood, evidence from other forms of renal fibrosis suggests that a crucial common pathway is a disturbance in the control of extracellular matrix (ECM) turnover. While this control is complex, one cytokine, transforming growth factor beta (TGF β), has been consistently shown to play a vital role.

TGF β has been studied extensively in experimental models of renal disease and increased expression and shown to be associated with histological changes of fibrosis [22]. In transplantation, there is much circumstantial evidence to support its role in CAN [8], however,

much about its overall significance in the process remains unanswered. One of the reasons for this is that it does play an immunosuppressive role, and, therefore, some of the up-regulation after transplantation may be beneficial [12].

As yet there have been no reports examining TGF-β expression in the same kidney sequentially from before transplantation and further into the early post-transplant period. This is of some importance, because it is likely that genotypic differences mean that individual recipients have different baseline levels that are related to their susceptibility to fibrosis [1]. Comparison between patients may, therefore, be unreliable. A greater understanding of the natural history of TGF-β expression after renal transplantation would put into a better context the changes that are found during disease processes such as CAN.

We previously studied the expression of active TGF β after renal transplantation using an immunofluorescence technique [14]. The aim of the present study is to quantify the changes in TGF-β expression after transplantation using protocol biopsies at pre-perfusion, at 1 week and at 6 months after transplantation.

Patients and methods

A consecutive series of 40 patients undergoing renal transplantation underwent protocol biopsies. Baseline biopsies (pre-perfusion) were taken from each kidney at the time of transplantation, and subsequently tru-cut biopsies were taken using ultrasound guidance at 1 week and at 6 months after transplantation. Patients whose grafts had failed prior to a 6-month protocol biopsy were not included in the study. All patients underwent regular clinic follow-up, and renal function was quantified by use of the isotope glomerular filtration rate (GFR) measured by ^{51}Cr-EDTA at 6 and 12 months after transplantation. For immunosuppression we implemented a dual therapy with calcineurin inhibitor (cyclosporine or tacrolimus) and prednisolone. Patients with kidneys from non-heart-beating donors received reduced doses of calcineurin inhibitors and azathioprine (triple therapy). No patients were administered angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptor antagonists. Biopsy specimens were all assessed by an experienced histopathologist (PNF) and graded according to Banff criteria [16].

TGF-β immunofluorescence staining

Paraffin-processed tissue sections were de-waxed and re-hydrated before being incubated with 'blocking' serum (normal human serum 1/15 and normal rabbit serum 1/5 in Tris-buffered saline (TBS) pH 7.6) for 2 h at 4 °C. This was followed by incubation with the primary antibody, chicken anti-human TGF β1 (RD systems) at 1/100 in blocking serum, overnight at 4 °C. The specificity of this primary antibody had previously been shown by its capacity to block the biological activity of TGF β in an epithelial-cell proliferation assay [13]. After having been washed in TBS for 10 min, sections were incubated for 2 h at 37 °C with rabbit anti-chicken IgG conjugated to fluorescein isothiocyanate at 1/400 in blocking serum. Sections were then washed for a further 10 min in TBS and mounted in fluorescence mounting medium (DAKO, Ely, Cambs., UK). Matched negative controls were prepared by replacement of the primary antibody with normal anti-chicken IgG.

Sections were analysed by semi-quantitative scanning laser confocal microscopy as described previously [17]. Data were expressed as the ratio of mean fluorescence over the selected area of experimentally stained tissue (excluding the tubule lumen) to the corresponding value in control sections.

Statistics

Values of TGF-β staining intensity over time were compared using the Wilcoxon signed rank test. Comparisons between different groups were made with the Kruskal-Wallis test for comparison of non-parametrically distributed variables.

Results

Details of the patients studied are shown in Table 1; those of the histological findings in the protocol biopsies in Table 2. Histological results were mostly normal, however occasional sub-clinical rejection was found. Over 25% of the 6-month biopsies showed evidence of fibrosis.

The most striking finding was the highly significant increase in active TGF-β staining 6 months after transplantation (Fig. 1). When the levels of the individual patients were plotted, 34 showed an increase in TGF β over time and only six a drop (data not shown). The TGF-β levels both at pre-perfusion and 1 week after transplantation were similar and did not show a large range. TGF-β levels did not seem to be affected by donor type (Fig. 2). Although there was only a small number of patients in this study, an attempt was made to determine whether any of the established prognostic factors for graft outcome were related to the rise in TGF β. Only delayed graft function (DGF) seemed to be relevant; patients with DGF displayed increased TGF-β expression at both 1 week and at 6 months, when compared with those who did not, however, neither of these results quite reached significance (Fig. 3, 4). Six patients developed acute rejection in the first 6 months after transplantation; they did not have higher levels of TGF β at 6 months (Fig. 5). The type of immunosuppressive drug administered did not affect 6-month

Table 1 Patient details ($n=40$) (*CAD* cadaveric donor, *NHBD* non-heart-beating donor, *LRD* living related donor, *CIT* cold ischaemia time)

Characteristic	
Mean donor age in years	42.5 (14.2)
Mean recipient age in years	44 (12.7)
Gender ratio (M:F)	24:16
Donor type (CAD; NHBD; LRD)	17; 14; 9
Mean CIT	12.3 (8.0)
DGF	14/40 (35%)
AR by 6 months	6/40 (15%)

Table 2 Histological findings in protocol biopsies (according to the Banff 1997 classification)

Diagnosis	Biopsy		
	Pre-perfusion	1 week	6 months
Normal	36	21	26
Acute tubule necrosis	0	13	0
Borderline rejection	0	4	3
AR	0	2	0
CAN	4	0	11

TGF-β levels either (Fig. 6), and there was no correlation with drug levels for either agent (data not shown). There was no correlation between TGF-β levels at any time-point and GFR at 6 or 12 months after transplantation.

Discussion

This study demonstrates increased levels of active TGF β in renal transplants at 6 months when compared with baseline pre-perfusion levels. As the study was carried out using protocol biopsies, changes were likely to be due to transplantation rather than to any particular condition for which the biopsies had been taken. This study comprises a relatively small number of patients and so does not have the power to demonstrate differences between patient groups after transplantation. Furthermore, it does not show any influence of TGF-β levels on prognosis. This will need larger studies.

There have been problems with the study of TGF-β expression in biopsy specimens because of uncertainties about whether staining distinguished between active and latent forms. The antibody used in this study has been shown to react only with active TGF β; this study was therefore carried out optimally [13].

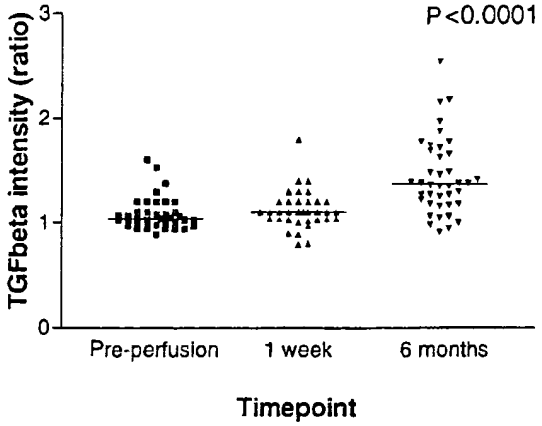

Fig. 1 Changes in TGF-β expression after transplantation

Although several human studies have reported increases in the expression of TGF β in CAN [15, 18, 19], none of these drew comparisons with stable post-transplant patients. Hence, it is not clear whether TGF β is truly involved in the process, or if it is an innocent bystander.

There have been few studies of renal TGF-β expression after transplantation in stable recipients. Lantz et al. included the biopsies of seven stable transplant recipients 6–24 months after transplantation in their series of 28 biopsies stained for TGF β [10]. Those showed definitely increased staining when compared with normal controls, measured in a semi-quantitative manner on a scale of + to + + +. No difference was shown between stable transplants and those with acute rejection (AR) or CAN. The only other study of TGF-β expression in stable patients after transplantation measured its levels in plasma. [5] There was a definite increase in the 17 transplant patients when compared with 43 healthy controls ($P=0.0004$). Again, no difference was determined between the stable transplants and those with AR or CAN. These findings are in agreement with those in the current study, which shows that while there was a definite increase in TGF β at 6 months, levels were not affected by established risk factors for CAN.

There is a very good reason for increased TGF-β levels in post-transplant patients, as it has an immunomodulatory role and is likely to be significant for reducing the extent of the reaction against the allograft [12]. It also seems to be up-regulated by ischaemia and to have a protective role [11]; indeed this may explain the increased levels found in patients with DGF in this series. As well as potential beneficial actions however, TGF β does play a role in fibrosis, which has been described as its dark side [2]. Possibly, certain patients are more prone to the unwanted effects of TGF β, and an interesting finding with regard to this has been that

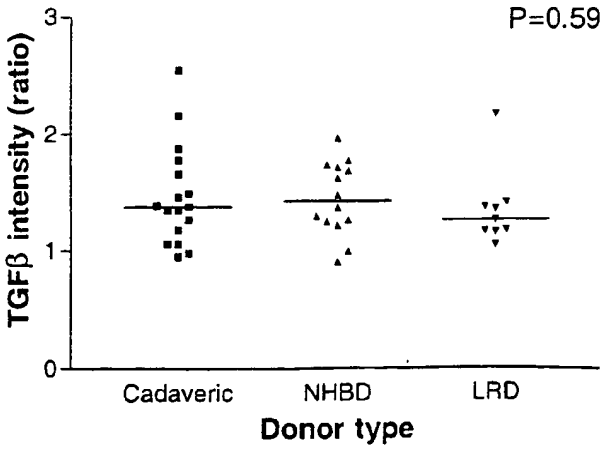

Fig. 2 Active TGF-β expression 6 months after transplantation, according to donor type

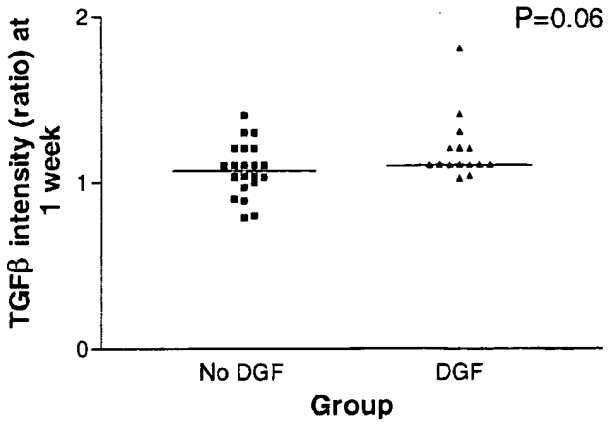

Fig. 3 Effect of DGF on active TGF-β expression 1 week after transplantation

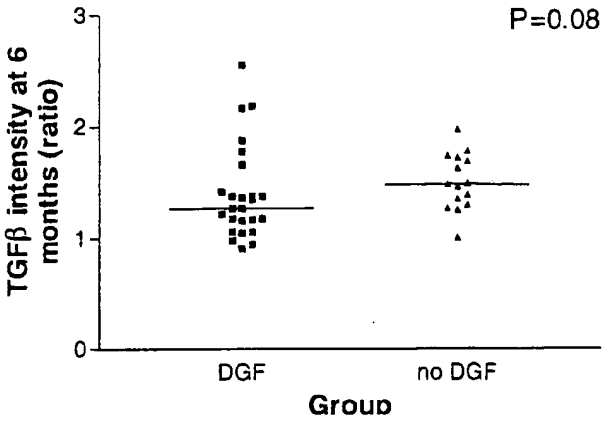

Fig. 4 Effect of DGF on active TGF-β expression 6 months after transplantation

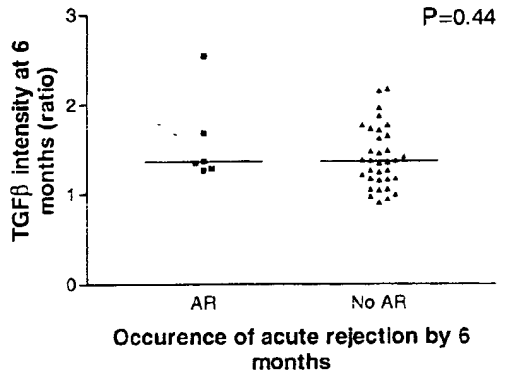

Fig. 5 Effect of AR on active TGF-β expression 6 months after transplantation

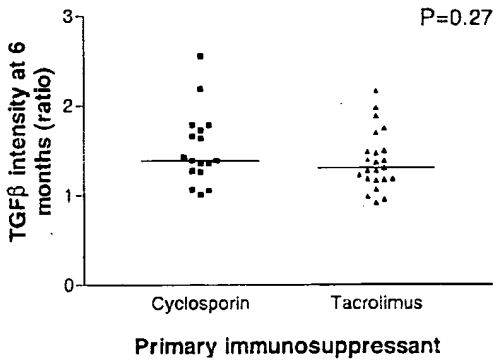

Fig. 6 Active TGF-β expression 6 months after transplantation, according to primary immunosuppressant drug

there are various genotypes of TGF β, with differing propensities for developing fibrosis [1].

The calcineurin inhibitors cyclosporine and tacrolimus induce TGF β [20, 21], and this has been proposed as a mechanism of action [9]. There has been much interest in differences between the two drugs, and indeed, we have previously shown that cyclosporine induced TGF β to a greater extent in biopsies taken for diagnostic purposes. A study using protocol biopsies after liver transplantation also showed this, and thus it is surprising that there was no difference found in the current study. However, all these studies comprise relatively small numbers, and so they are probably not conclusive. There have certainly been other studies that could not find a difference in the effects of the two drugs on TGF β, either [7]. ACE inhibitors have been shown to have a down-regulatory effect on TGF-β expression [3]. While there were no patients on these drugs in this study, they may have had a confounding effect when drug groups in other studies were compared. The use of angiotensin II receptor blockers therapeutically to reduce TGF-β levels after renal transplantation is an interesting concept that is being explored [4].

A correlation between increased TGF-β levels and rate of decline in renal function has been shown by Cuhaci et al. [6]. In this study of forty patients, TGF-β levels were graded as low or high in a qualitative manner. There was no correlation between TGF-β levels at 1 week and at 6 months and renal function at 6 months and at 1 year in this study. Obviously longer follow-up will allow a more detailed analysis to be made.

In conclusion, there still remains much to be learned about the importance of TGF β after renal transplantation, and its role needs to be defined more clearly before its manipulation for potential therapeutic gain is a practical option.

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